**The Role of O-GlcNAc in Liver Injury and Regeneration**

By


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**Abstract**

O-GlcNAcylation is a covalent attachment of a single N-acetyl glucosamine to a serine or threonine residue of a protein. Unlike other forms of protein glycosylation, there is no chain elongation in O-GlcNAcylation. O-GlcNAc transferase (OGT) adds O-GlcNAcase (OGA) removes O-GlcNAc from a protein. The process of O-GlcNAcylation is dynamic and has extensive crosstalk with protein phosphorylation. O-GlcNAcylation plays an important role in cell cycle progression, metabolic disorders, cancer, and responses to various stressors. The role of O-GlcNAc in liver injury and regeneration has not been clearly defined. Due to the wide array of cellular processes and pathologies that involve O-GlcNAc and the knowledge gap in liver injury and regeneration, we set out to determine the role O-GlcNAc plays in liver injury and regeneration.

APAP is the most widely used over-the-counter analgesic and antipyretic in the western world. Overdose of APAP leads to GSH depletion and centrilobular liver damage. First, we looked at the role of O-GlcNAc in response to APAP overdose in mice using both a decrease and an increase in O-GlcNAcylation. Hepatocyte specific deletion of OGT (OGT KO) in mice was used to decrease O-GlcNAcylation. OGT KO mice and WT mice were treated with 300 mg/kg APAP and the development of liver injury was studied over a time course of 0-24 hr. OGT KO mice showed dramatic decrease in liver injury compared to wild type mice. Serum ALT levels were markedly decreased in all time points in OGT KO mice and immunohistochemistry showed no centrilobular necrosis or cell death. OGT KO mice showed decrease in APAP-Cys protein adducts. There

was no difference in CYP2E1 protein levels or activity and initial GSH depletion between OGT KO and WT mice. There was rapid replenishment of GSH in OGT KO mice and induction of enzyme critical to GSH biosynthesis and recycling. Treatment with Thiamet-G (TMG), a potent inhibitor of OGA, was used to model a increase in O-GlcNAcylation. C57BL/6J mice were treated acutely with 400 mg/kg TMG 1.5 hr after APAP treatment. TMG treated mice showed an increase in APAP-induced liver injury as seen by Serum ALT immunohistochemistry. Acute TMG treatment also induced a prolonged JNK activation. Treatment with acute TMG did not affect hepatic CYP2E1 levels, APAP-protein adducts, and APAP-induced mitochondrial damage. Whereas GSH depletion was not different, GSH replenishment and GSH biosynthesis genes were lower in TMG-treated mice after APAP overdose.

Next, we examined the effects of OGT KO on liver regeneration after partial hepatectomy (PHX). WT mice had normal regeneration that subsided at 7 days post PHX. OGT KO mice showed normal regeneration up to 48 hr post PHX, but exhibited significant increase in liver regeneration from 5-14 days post PHX. OGT KO mice had higher liver to body weight ratio at 7 and 14 days and several pathways known to be critical in liver regeneration remained active out to 14 days post PHX. Indicative of an increase in proliferation, protein associated with cell cycle and cell cycle progression such as Cyclin D1, A2, B1, pRb, and CDK4 levels were all elevated at later time points. HNFα was decreased at 14 days, and several genes knows to be down regulated by HNF4a had increased fold change as seen in RNA-Seq data. Taken together, this data shows that OGT

KO increases liver regeneration after PHX and leads to a loss of termination of liver regeneration.

Overall, these studies show that O-GlcNAcylation plays a central role in liver injury and regeneration, and could potential lead to therapeutic interventions for APAP induce acute liver failure and liver cancer.

**Acknowledgments**

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# Chapter 1- Introduction

## 1.1 O-GlcNAc

N-acetyl glucosamine (O-GlcNAc) was discovered over 30 years ago, in 1983, by Drs. Torres and Hart at the Johns Hopkins School of Medicine (Torres and Hart 1984). Since this discovery, there have been many new and exciting findings in the field of O-GlcNAcylation, and this little single sugar-mediated posttranslational modification has proven to be quite a big player. O-GlcNAcylation is the dynamic covalent attachment of an O-GlcNAc moiety to a serine or threonine residue of nuclear, cytoplasmic and mitochondrial proteins (Zachara, O'Donnell et al. 2004, Slawson, Copeland et al. 2010). Unlike other forms of glycosylation there is no chain elongation of the sugar into more complex oligosaccharides and it is only found in the intracellular compartment, and thus is not found at the cell surface or in the extracellular space (Slawson and Hart 2011). O-GlcNAc cycling also differs from traditional forms of protein glycosylation in that it is dynamic, meaning there is rapid cycling of O-GlcNAc on and off proteins. To date, no consensus binding sequence or motif for O-GlcNAc has been identified; but that has not stopped the identification of over 4000 proteins that are modified by O-GlcNAc with the development of modern mass spectrometry (MS) techniques (Ma and Hart 2014).

O-GlcNAc is the end product of the hexosamine biosynthetic pathway (HBP) (Fig.1.1.1). Flux through the HBP is determined by glucose uptake in the cell. Glucose molecules that enter glycolysis are converted to glucose-6-phosphate by the enzyme hexokinase. Glucose-6-phosphate is then isomerized to fructose-6-phosphate by Glucose-6-phosphate isomerase, of which a portion

enters the HBP (Teo, Wollaston-Hayden et al. 2010). Around 2-3% of all cellular glucose enters the HBP and the final product of the pathway is the high-energy donor substrate UDP-GlcNAc (Slawson, Copeland et al. 2010, Bond and Hanover 2015). Flux through the HBP is controlled by a rate-limiting enzyme glutamine fructose-6-phosphate amidotransferase, or GFAT, and feedback inhibition through the production of UDP-GlcNAc. HBP it at a crucial junction of cell metabolism pathways including protein, carbohydrate, lipid, and nucleotide metabolisms; as well as energy changes due to the need of ATP required to run the pathway (Hanover, Krause et al. 2012). Because of its location downstream of so many processes central to the function of the cell, O-GlcNAc and flux through the HBP are often thought to be a nutrient sensor (Butkinaree, Park et al. 2010). The availability of glucose can control the amount of O-GlcNAcylation seen on intracellular proteins, and in turn O-GlcNAcylation is sensitive in changes to glucose metabolism. It has been shown that O-GlcNAc levels attenuate insulin signaling and contribute to the mechanism by which increased flux through the HBP leads to insulin resistance in adipocytes (Vosseller, Wells et al. 2002).

O-GlcNAc cycling also differs from traditional forms of protein glycosylation in that it is dynamic, meaning there is rapid cycling of O-GlcNAc on and off proteins. The actual process of O-GlcNAcylation, the addition of O-GlcNAc to the protein backbone using UDP-GlcNAc as a substrate, is performed by two specific enzymes O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA). OGT is located on the X chromosome and is a highly conserved protein that

exists in mammals in three isoforms that only differ in the amount of tetratricopeptide repeats (TPRs)- the nucleocytoplasmic (ncOGT), the short isoform (sOGT) and the mitochondrial form (mOGT) (Shafi, Iyer et al. 2000, Lazarus, Love et al. 2006, Hanover, Krause et al. 2012). OGA exists as two isoforms; the long (OGA-L), which contains a histone acetyl transferase domain (HAT), and the short (OGA-S) (Bond and Hanover 2015) (Fig.1.1.2). OGT is the only enzyme capable of adding O-GlcNAc, and conversely OGA is the only enzyme that removes O-GlcNAc. This specificity of enzymes for addition and removal also distinguishes O-GlcNAcylation from other forms of glycosylation.

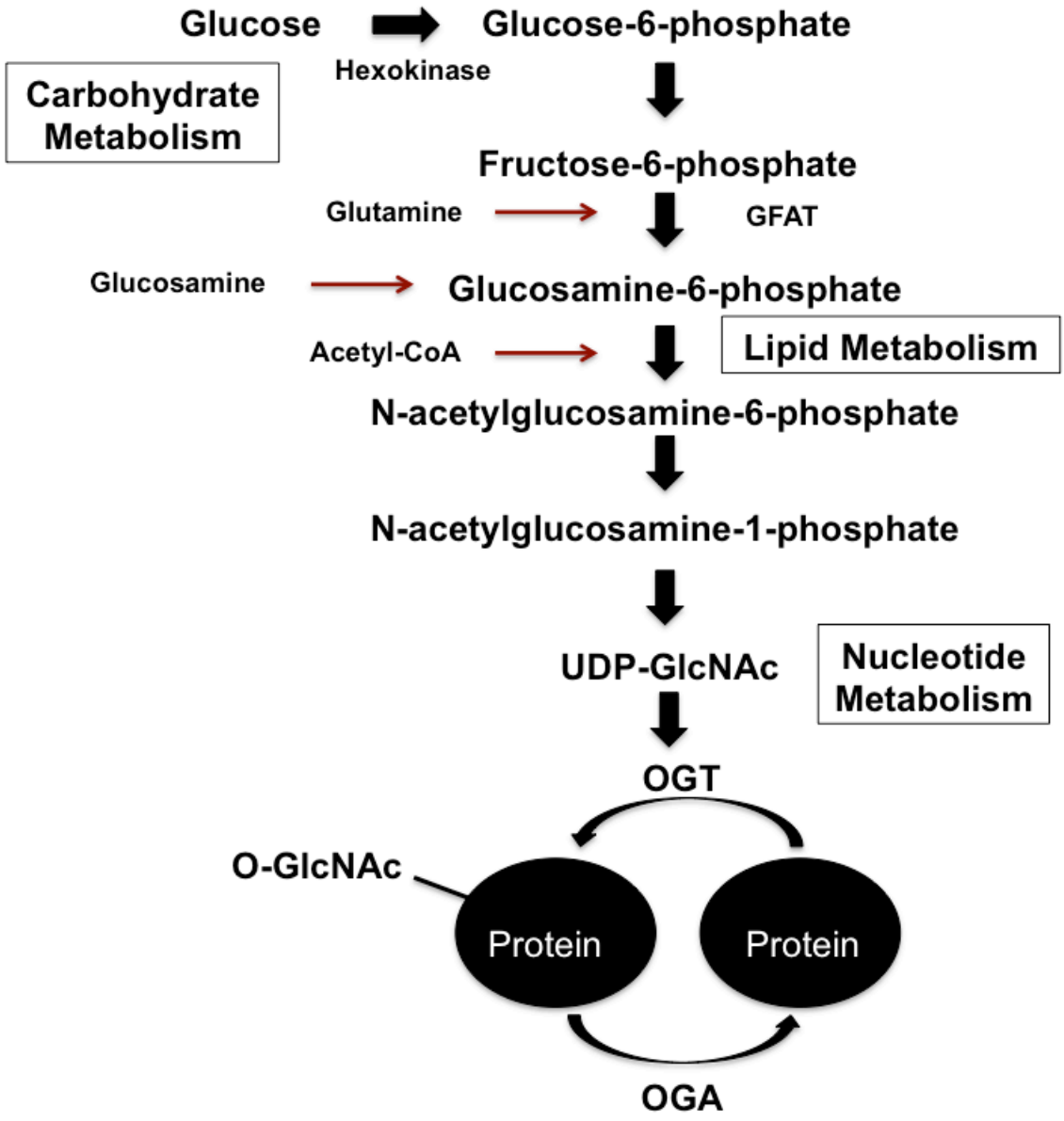



**Fig.1.1.1.** A schematic representation of the hexosamine biosynthetic pathway (HBP) and how it integrates many other cellular processes. Glucose enters the pathway to form the end product UDP-GlcNAc.

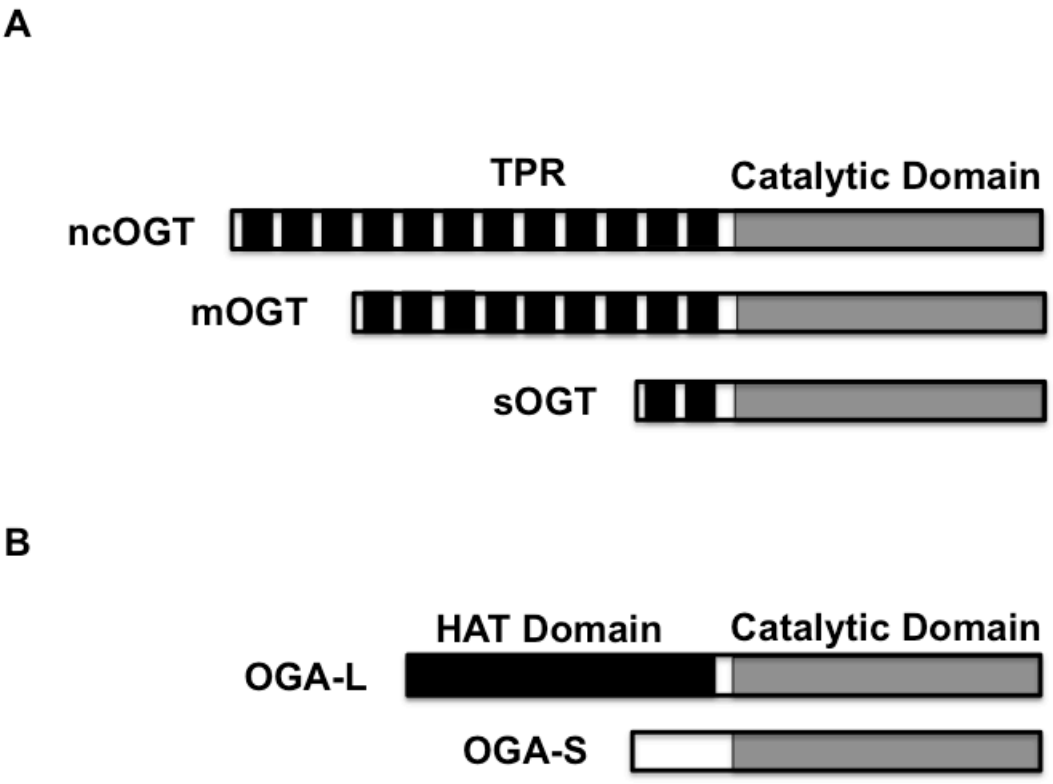



**Fig.1.1.2.** (A) The three isoforms of OGT showing the variable TPR repeats- ncOGT (12 TPRs), mOGT (9 TPRs) and sOGT (2 TPRs). (B) The two isoforms of OGA- OGA-L with a HAT domain and OGA-S without a HAT domain.

Extensive studies show that O-GlcNAc has cross talk with protein phosphorylation in many signaling pathways (Wang, Gucek et al. 2008, Zeidan and Hart 2010). O-GlcNAcylation sites have been found to be identical or proximal to the same serine and threonine phosphorylation sites used by kinases (Hart, Slawson et al. 2011). When O-GlcNAc is elevated in mammalian cells, 70% of phosphorylation sites are affected, making it extremely important in signaling cascades and enzyme function (Wang, Gucek et al. 2008). The relationship between O-GlcNAcylation and protein phosphorylation is so interlinked that both OGT and OGA often occur in complexes that contain both kinases and phosphatases (Slawson, Lakshmanan et al. 2008, Zeidan and Hart 2010). Because of this close relationship with protein phosphorylation, O-GlcNAcylation can affect many cellular processes regulated by phosphorylation.

The O-GlcNAcylation of proteins is dynamic and often times a response to cellular stress, where a wide variety of cellular stresses lead to a rapid increase in the O-GlcNAcylation of nuclear and cytoplasmic proteins (Zachara, O'Donnell et al. 2004). It has also been shown that mitogenic activation of T lymphocytes induces a rapid change in both nuclear and cytosolic O-GlcNAcylation (Kearse and Hart 1991). O-GlcNAc levels are important regulators of the cell cycle and are involved in cell cycle progression, controlling mitotic phosphorylation, regulation of cyclin expression, and cytokinesis (Slawson, Zachara et al. 2005). O-GlcNAc plays a very important role in many cellular processes including organ development (Hanover, Krause et al. 2012), cancer pathogenesis (Slawson and Hart 2011), stress response (Chatham and Marchase 2010) and tau

phosphorylation (Yu, Zhang et al. 2012). It has been shown that O-GlcNAcylation is crucial for embryonic development. For example, deletion of OGT leads to loss of embryonic stem cell viability (Shafi, Iyer et al. 2000). O-GlcNAc also plays a key role in many cancers, including breast, lung, colon, and prostate cancer (Slawson and Hart 2011, Ferrer, Sodi et al. 2016). O-GlcNAcylation has also been shown to be critical in Alzheimer's disease (Zhu, Shan et al. 2014), cardiovascular disease (Zachara 2012), and the metabolic syndromes and diabetes (Ma and Hart 2013). A brief list of target organs or systems affected by O-GlcNAc is shown in Fig.1.1.3 to outline the wide-reaching effects of O-GlcNAc on cellular function.

O-GlcNAc has also been shown to be involved in mitochondrial function and oxidative stress (Love, Kochan et al. 2003, Tan, Villar et al. 2014). Altering the cycling of O-GlcNAc also has profound effects on the mitochondrial function and the respiratory chain (Tan, Villar et al. 2014). In mice, a knockout of OGT leads to impaired mitochondrial function, increased reactive oxygen species levels and activation of the main antioxidant defense gene NRF2 (Tan, McGreal et al. 2017). The relationship between O-GlcNAc and oxidative stress could prove to be of critical importance after exposure to a toxicant, such as acetaminophen, that depletes antioxidants, specifically glutathione (GSH).

O-GlcNAc is also important for the cell cycle and cell cycle progression. Elevated O-GlcNAc is a key regulator of mitotic phosphorylation, leads to cell growth delays of many cell types, and the overexpression of either OGT or OGA disrupts cyclin periodicity and causes improper cytokinesis (Slawson, Zachara et

al. 2005). The coordinated cycling of O-GlcNAc by OGT and OGA is required to regulate mitotic spindle formation, where an increase in O-GlcNAc cycling leads to improper mitotic spindles formation (Tan, Caro et al. 2013). In mouse embryonic fibroblasts, the deletion of OGT leads to an increase in the expression of the cyclin inhibitor p27, growth delays and eventual cell death (O'Donnell, Zachara et al. 2004). Similarly, a stable knockdown of OGA in HeLa cells causes a prolonged M phase of the cell cycle leading to improper cytokinesis, shortened spindle chromatids and increased multi-polar spindles (Lanza, Tan et al. 2016). The alteration in the cell cycle shows that maintaining O-GlcNAc homeostasis is a critical component of proper cell cycle progression and completion.

There are several different ways to modulate cellular O-GlcNAc levels. Pharmacologic agents, such as PUGNAc, Streptozotacin and Thiamet G (TMG) have been shown to inhibit the enzyme OGT and increase cellular levels of O-GlcNAc (Macauley and Vocadlo 2010). TMG treatment has been shown to increase O-GlcNAcylation and decrease phosphorylation of tau proteins, a hallmark of Alzheimer's disease (Yu, Zhang et al. 2012). This has led to the development of TMG as a possible treatment for Alzheimer's disease.

Studies show that increasing flux through HBP can increase the levels of O-GlcNAc in the cell. Over the counter glucosamine has been shown to increase cellular O-GlcNAc levels and flux through the HBP (Tannock, Kirk et al. 2006). Glucosamine is able to bypass glutamine fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme of HBP (Teo, Wollaston-Hayden et al. 2010). Previous studies with glucosamine treatment have shown that supplementation

of diet or addition to drinking water (Tannock, Kirk et al. 2006) daily intraperitoneal injection (IP) (Song, Kang et al. 2014) and oral gavage (Carames, Kiosses et al. 2013) can increase cellular levels of O-GlcNAc. Modulation of O-GlcNAc can also be done at a gene level by selective knockout of genes involved in the cycling of O-GlcNAc, specifically OGT. An OGT floxed mouse ($OGT^F$) has been developed and the resulting knockout of OGT leads to a decrease in the levels of O-GlcNAc (Shafi, Iyer et al. 2000). The generation of an OGA knockout mouse shows that a deletion of OGA leads to defects in metabolic homeostasis and eventually obesity and insulin resistance (Keembiyehetty, Love et al. 2015). Due to the general ease by which cellular O-GlcNAc levels can be modulated and the great number of cellular processes O-GlcNAcylation is involved in, the role of O-GlcNAc needs to be further investigated.

| Organ or System | Effect | Reference |
|---|---|---|
| **Brain** | Regulates Tau phosphorylation | (Yu, Zhang et al. 2012) |
| | Alzheimer's Disease | (Zhu, Shan et al. 2014) |
| **Eye** | Diabetic Retinopathy | (Semba, Huang et al. 2014) |
| **Heart** | Increased O-GlcNAc protects cardiomyocytes from injury | (Champattanachai, Marchase et al. 2008) |
| | Increased O-GlcNAc inhibits inflammation in acute arterial injury | (Xing, Feng et al. 2008) |
| | O-GlcNAc is indispensable in the failing heart | (Watson, Facundo et al. 2010) |
| **Liver** | Tumor recurrence in HCC | (Zhu, Zhou et al. 2012) |
| **Colon** | Increased levels of O-GlcNAc in colon cancer cells | (Yehezkel, Cohen et al. 2012) |
| **Adipocytes** | Increased O-GlcNAc results in insulin resistance | (Vosseller, Wells et al. 2002) |
| **Circulatory/ Blood** | Increased O-GlcNAcylation is associated with increased insulin sensitivity in blood | (Myslicki, Shearer et al. 2014) |

**Fig.1.1.3.** A brief list of some of the organs and systems affected by O-GlcNAc biology, showing the wide-ranging processes that involve O-GlcNAcylation.

## 1.2 Acetaminophen-induced Acute Liver Failure

Acetaminophen (APAP) is a safe over the counter analgesic and anti-pyretic drug when used at therapeutics doses, but it can cause hepatotoxicity when taken in excess. In the United States, APAP overdose is the leading cause of drug induced acute liver failure (ALF), and APAP overdose accounts for more than 100,000 calls to poison control centers, 56,000 emergency room visits, 2,600 hospitalizations and 500 deaths (Nourjah, Ahmad et al. 2006). APAP overdose cases can be divided into two categories; unintentional overdose, where the patient takes a large amount of APAP because they are unaware that several products contain APAP or they are not getting the desired pain relief, and intentional suicidal overdose (Lee 2004). The mechanism of APAP toxicity has been extensively studied over the decades, but the treatment options are still limited. N-acetylcysteine, a glutathione precursor, is the only recognized pharmacological intervention, but it has only been shown to be effective when given in the early stages after APAP overdose (Yang, Miki et al. 2009). Ultimately, patients who overdose on APAP may be subject to a liver transplantation. Liver transplantation is complicated by lack of donor availability, graft rejection, cost and late presentation with rapid decline (Lee 2012).

The majority of a dose of APAP is either glucuronidated or sulfated by phase II enzymes and excreted safely. A small portion of the dose is converted to the reactive metabolite N-acetyl-benzoquinone imine (NAPQI) by cytochrome P450 enzymes, with CYP2E1 being the primary enzyme in rodents. NAPQI can be conjugated to glutathione and excreted, but the glutathione stores are rapidly

depleted in an APAP overdose scenario. Once glutathione is depleted, NAPQI can bind to proteins to form protein adducts (McGill and Jaeschke 2013). If glutathione is depleted prior to dosing with APAP, there is impairment of the antioxidant defense system, and it has been show that providing N-acetylcysteine during the initial injury period is protective against APAP-induced liver damage (James, McCullough et al. 2003, Saito, Zwingmann et al. 2010). It is known that APAP-induced liver injury involves a cascade of events following bio-activation of APAP to NAPQI including increased formation of reactive oxygen and reactive nitrogen species, JNK activation, mitochondrial damage and ultimately cellular necrosis (Jaeschke and Bajt 2006, Jaeschke, McGill et al. 2012). This cascade of events ultimately leads to ALF (Fig.1.2.1).

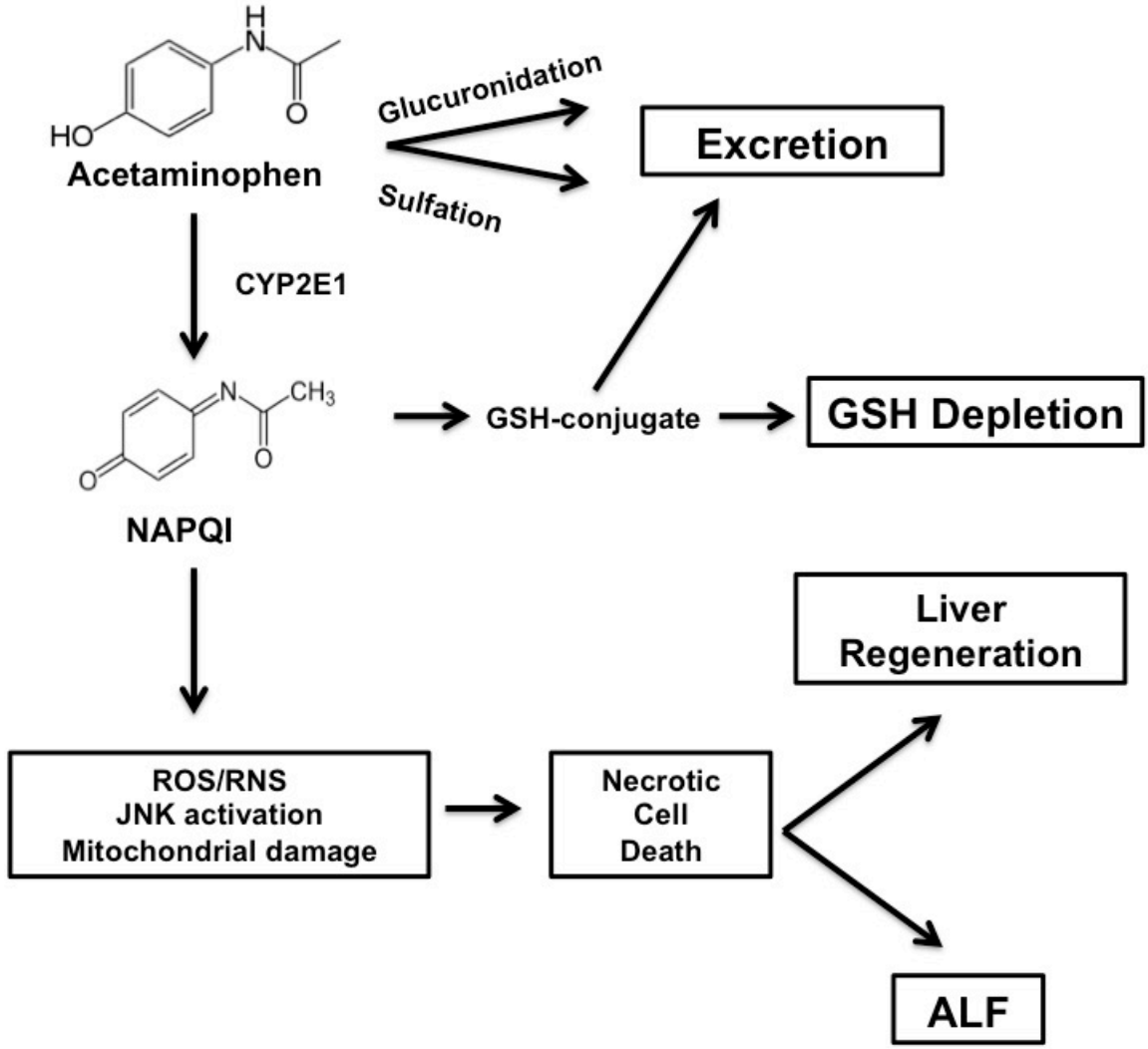



**Fig.1.2.1.** A graphic representation of APAP metabolism leading to acute liver failure (ALF).

A major factor in the outcome on drug induced ALF is the innate ability of the liver to regenerate itself after insult or injury. The liver regenerates in response to hepatotoxicants such as APAP, carbon tetrachloride, chloroform, and thioacetamide (Mehendale 2005). After insult by APAP, the regeneration of the liver follows the dose response where increasing the dose increases the liver injury and there is also a proportionate increase in liver regeneration. This is followed up to a threshold dose, beyond which the liver loses the ability to regenerate and injury progresses. This principle of dose response is shown using a novel incremental dose model. Mice treated with 300 mg/kg APAP showing injury progression accompanied by marked increase in regeneration, while mice treated with 600 mg/kg APAP showed injury progression but had substantial inhibition of liver regeneration (Bhushan, Walesky et al. 2014). Liver regeneration after drug overdose is an essential factor in determining the final patient outcome and understanding the mechanisms behind the liver's ability to regenerate is paramount to discovering new treatment options.

## 1.3 Liver Regeneration

The ability of the liver to regenerate after insult or partial resection has been known since the times of the ancients Greeks and can be seen in the telling of the myth of Prometheus. Liver regeneration is studied by two methods, partial hepatectomy (PHX) and insult by hepatotoxicants such as APAP. While both models of liver regeneration are useful, there are some distinct differences. Toxicant induced liver injury produced by APAP involves necrosis and

inflammation; which are absent in PHX. In PHX, all surviving hepatocytes undergo proliferation. In APAP induced liver failure, only the hepatocytes surrounding the necrotic zone regenerate to restore the liver tissue.

As mentioned previously, the liver regenerates in response to partial hepatectomy (PHX) where approximately 70% of the liver is removed surgically. The events of liver regeneration after PHX occur in an orderly fashion over a time course from within the first 5 minutes to 7 days after surgery, with the hepatocytes in the remaining liver mass undergoes proliferation to restore the removed tissue (Michalopoulos 2007). All four major cells types of the liver undergo regenerations to restore the lost cell mass from PHX. Hepatocytes begin with one round of DNA replication with a peak at 24 hours after PHX, and are followed by biliary ductal cells and Kupffer cells that peak at 48 hours post PHX and sinusoidal endothelial cells that peak at 4 days post PHX (Michalopoulos and DeFrances 1997).

There are two distinct schools of thought on how liver regeneration is initiated. The first is the priming and progression model developed by Nelson Fausto. Priming and progression involves cytokines, such as TNFα and IL-6, that prime the liver to leave G0 and enter G1 phase of the cell cycle; and growth factors such as HGF, EGF and TGF that cause the progression from G1 to M phases of the cell cycle (Fausto, Campbell et al. 2006). The second paradigm of liver regeneration is primary and secondary mitogens created by George Michalopoulos. Primary or direct mitogens, such as hepatocyte growth factor (HGF) and epidermal growth factor (EGF), are able to induce proliferation in

serum free media (Block, Locker et al. 1996). Secondary mitogens are all other molecules that assist in hepatocyte proliferation, but cannot induce proliferation on their own. These secondary mitogens include TNF (Webber, Bruix et al. 1998), norepinephrine (Cruise, Houck et al. 1985), and estrogens (Ni and Yager 1994).

Although there is much known about the initiation of liver regeneration and the signaling cascade that follows PHX, there is very little known about the signal that terminates liver regeneration. Although the mechanism is not completely known, many factors have been implicated in the termination of liver regeneration. Previous studies have shown that the nuclear receptor HNF4α can suppress hepatocyte proliferation, and its deletion can result in increased hepatocyte proliferation and cancer (Bonzo, Ferry et al. 2012, Walesky, Gunewardena et al. 2012, Walesky, Edwards et al. 2013). ILK deletion in hepatocytes leads to increase in post-PHX liver mass, leading to the conclusion that the extra cellular matrix mediated signaling driven by ILK is critical in the termination of liver regeneration (Apte, Gkretsi et al. 2009). Hippo/Yap signaling is known to help maintain organ size and development (Zhao, Tumaneng et al. 2011). Over activation of Yap, i.e. the loss of phosphorylation of Yap, by Mst1/2 deficiency leads to massive liver overgrowth and cancer (Zhou, Conrad et al. 2009). There are many factors that can alter the state of termination during liver regeneration, but more work is needed to elucidate all the contributing factors.

## 1.4 Purpose and Aims

O-GlcNAcylation plays such an important role in a wide variety of cellular processes and O-GlcNAcylation is thought to be important in the treatment and pathogenesis of many diseases including Alzheimer's disease (Macauley and Vocadlo 2010), recurrence of hepatocellular carcinoma (HCC) after liver transplantation (Zhu, Zhou et al. 2012) and metabolic disorder and diabetes (Wang, Park et al. 2009, Myslicki, Shearer et al. 2014). However, the role of O-GlcNAcylation in APAP-induced acute liver failure and liver regeneration after partial hepatectomy has not been studied. This is important because of possible drug interactions between a wide variety of medications that contain APAP and over the counter supplements such as Glucosamine that can increase cellular O-GlcNAc levels. It is highly likely that supplements that increase O-GlcNAc levels may affect the safety of APAP. Furthermore, because O-GlcNAcylation is a ubiquitous process, it is highly likely to be involved in hepatocellular injury and regeneration, which has not been investigated. Thus, these studies are important both from drug safety and a basic biology standpoint.

The long-term goal of this project is twofold; 1- to clearly define the role O-GlcNAc plays in acute liver injury after APAP overdose and 2- to clearly define the effects of OGT knockout on the liver after partial hepatectomy.

Specific Aim #1: Determine the role of O-GlcNAcylation after APAP overdose

- Determine the effects of an increase in O-GlcNAcylation on liver injury by treating C57Bl/6J mice with 300 mg/kg of APAP followed 1.5 hr later with 400 mg/kg TMG.
- Determine the effects of chronic O-GlcNAcylation on liver injury treating C57Bl/6J mice with 50 mg/kg TMG every other day for 15 days followed by 300 mg/kg of APAP.
- Determine the effects on liver injury after a decrease in O-GlcNAcylation using $OGT^F$ mice. Induce a knockout of OGT using AAV8.TBG.Cre virus in hepatocytes and treat mice 10 days later with 300 mg/kg APAP.

We hypothesize that any alteration if O-GlcNAcylation will have profound effects on the safety of APAP, with an increase in global O-GlcNAc increasing liver damage after APAP and a decrease in global O-GlcNAcylation leading to a decrease in APAP induced liver damage.

Specific Aim #2: Determine the role of O-GlcNAcylation in liver regeneration after partial hepatectomy (PHX)

- Determine the effects of a decrease in O-GlcNAcylation on the livers ability to regenerate after PHX using $OGT^F$ mice. Induce a knockout of OGT using AAV8.TBG.Cre virus in hepatocytes and perform PHX 10 days later.

We hypothesize that a decrease in O-GlcNAcylation will adversely alter liver regeneration after PHX.

# Chapter 2- Modulation of O-GlcNAc levels in the liver impacts acetaminophen-induced liver injury by affecting protein adduct formation and glutathione synthesis

## 2.1 Abstract

Overdose of acetaminophen (APAP) results in acute liver failure. We have investigated the role of a post-translational modification of proteins called O-GlcNAcylation, where the O-GlcNAc transferase (OGT) adds and O-GlcNAcase (OGA) removes a single β-D-N-acetylglucosamine (O-GlcNAc) moiety, in the pathogenesis of APAP-induced liver injury. Hepatocyte specific OGT knockout mice (OGT KO), which have reduced O-GlcNAcylation, and WT controls were treated with 300 mg/kg APAP and the development of injury was studied over a time course from 0-24 hr. OGT KO mice showed a dramatic decrease in liver injury as compared to WT mice. Whereas, there was no difference in CYP2E1 activity or GSH depletion following APAP treatment in OGT KO mice, replenishment of GSH and induction of GSH biosynthesis genes were significantly faster in WT mice. Next, male C57BL/6J mice were treated Thiamet-G (TMG), a specific inhibitor of OGA to induces O-GlcNAcylation, 1.5 hr after APAP administration and the development of liver injury was studied over a time course of 0-24 hr. TMG treatment resulted in significant increase in hepatic O-GlcNAcylation. TMG-treated mice exhibited significantly higher APAP-induced liver injury as indicated by an increase in serum transaminase levels and histopathological analysis, as well as a prolonged JNK activation. Treatment with TMG did not affect hepatic CYP2E1 levels, APAP-protein adducts, and APAP-induced mitochondrial damage. Whereas GSH depletion was not different, GSH replenishment and GSH biosynthesis genes were lower in TMG-treated mice after APAP overdose. Taken together, these data indicate that induction in

cellular O-GlcNAcylation exacerbates APAP-induced liver injury via dysregulation of hepatic GSH replenishment response.

## 2.2 Introduction

Overdose of acetaminophen (APAP) is the most common cause of acute liver failure (ALF) in the developed countries (Nourjah, Ahmad et al. 2006, Lee 2008). Treatment options for APAP-induced ALF are extremely limited (Lee 2008). Despite extensive investigations the mechanisms of APAP-induced liver injury are not completely clear (Jaeschke and Bajt 2006, Han, Shinohara et al. 2010, Jones, Lemasters et al. 2010, Jaeschke, McGill et al. 2012). In recent years, several types of post-translational modifications (PTM), which modulate pathophysiological processes via modifying protein function, have been described (Schrattenholz, Soskic et al. 2010, Anbalagan, Huderson et al. 2012). However, the role of PTMs in APAP-induced liver injury has not been investigated.

One such PTM is O-GlcNAcylation where a single β-D-N-acetylglucosamine (O-GlcNAc) moiety is added to serine/threonine residues of intracellular proteins (Slawson and Hart 2003, Hart, Housley et al. 2007, Slawson and Hart 2011). An enzyme called O-linked N-acetylglucosamine transferase (OGT) catalyzes O-GlcNAcylation using UDP-GlcNAc as the sugar donor (Fig.2.4.2A). In contrast, the enzyme O-GlcNAcase (OGA) removes O-GlcNAc from the proteins. Extensive studies have demonstrated that O-GlcNAcylation of proteins results in significant changes in protein function and is involved numerous cellular and pathological processes including organ development (Hanover, Krause et al. 2012, Liu, Li et al. 2012), cancer pathogenesis (Slawson and Hart 2011), the stress response (Chatham and Marchase 2010, Martinez,

Dias et al. 2017) and cellular proliferation (Slawson, Zachara et al. 2005, Slawson, Copeland et al. 2010). Recently, increased levels of O-GlcNAc have been shown to control tau phosphorylation in the mammalian brain, which could lead to a possible treatment for Alzheimer's disease (Macauley and Vocadlo 2010). Additionally, modulation of O-GlcNAc has been investigated as a possible treatment for many diseases (Macauley, Shan et al. 2010, Macauley and Vocadlo 2010). However, the effect of changes in hepatic O-GlcNAc levels on APAP toxicity has not been investigated.

In this study, we investigated the effect of O-GlcNAcylation on APAP toxicity using two approaches to modulate cellular O-GlcNAc levels *in vivo* – a hepatocyte-specific OGT knockout mouse, which has significantly less O-GlcNAcylation and treatment with Thiamet-G (TMG), an inhibitor of OGA, to increase the levels of O-GlcNAc. Our data indicate that the alteration of O-GlcNAc levels in the liver can have profound effects on APAP-induced liver injury.

## 2.3 Materials and Methods

*Animal and Tissue Preparation*

All animal studies were approved by and performed in accordance with the Institutional Animal Care and Use Committee at the University of Kansas Medical Center. OGT-floxed mice (Shafi, Iyer et al. 2000) were a gracious gift from Natasha Zachara at The Johns Hopkins School of Medicine and were used to generate hepatocyte specific OGT knockout mice (OGT KO). Two to three month old male OGT-floxed mice (n=3-5 mice per group per time point) were treated with Cre-recombinase driven by thyroxin binding globulin promoter carried by the AAV8 virus [AAV8.TBG.PI.Cre.rBG (Penn Vector Core, Philadelphia, PA)] at a $5E^{11}$ virus particles per mouse 10 days before dosing with APAP. OGT-floxed mice treated with an AAV8 carrying eGFP were used as control. For experiments with Thiamet-G (TMG) (SD Chemmolecules LLC, Owings Mills, MD), 2-3 old month male C57BL/6J mice (n=3 to 5 per group per time point) purchased from Jackson Laboratories (Bar Harbor, ME) were used. All mice were fasted overnight and treated with 300 mg/kg APAP (Sigma, St. Louis, MO). APAP was dissolved in warm 0.9% saline and was administered intraperitoneally (i.p.). In acute TMG treatment studies, mice were divided in two groups after APAP treatment and injected with either 0.9% saline or 400 mg/kg TMG dissolved in 0.9% saline, i.p., 1.5 hr after APAP treatment (Fig.2.4.1). In chronic TMG treatment studies, 2-3 month old male C57BL/6J mice were treated with 50 mg/kg TMG every other day for 2 weeks before APAP treatment 24 hr after the last TMG dose. In TMG dose finding studies, mice were fasted for 12 hr, treated

with either 400 or 800 mg/kg TMG dissolved in 0.9% saline and euthanized 18 hr after TMG treatment. Livers and blood samples were collected and processed as described before (Borude, Edwards et al. 2012, Walesky, Gunewardena et al. 2012). Briefly, mice were euthanized by cervical dislocation after anesthesia with isoflorane (Piramal Healthcare, Andhra Pradesh, India) at various time points over a time course of 0 to 24 hr following APAP treatment. Blood was collected via optic vein catheter and the liver was removed surgically. Liver samples were used for histopathology and serum ALT activities were measured as a marker of liver injury as described before (Borude et al., 2012).

*Staining procedures*

H&E staining was done on a Leica Autostainer XL and CV5030 cover slipper. Staining was conducted using 4 μm thick paraffin embedded liver samples as described previously (Borude et al., 2012). Oil Red O staining was performed using 8 μm frozen liver sections as described before (Borude et al., 2012). Necrosis scoring was performed as previously described (Bhushan, Walesky et al. 2014). Briefly, at least 5 high power fields (400x) were scored in H&E stained slides from 3-5 animals per group by an experienced pathologist. Slides were blinded to the observer. Scores were used for statistical analysis.

*Protein Isolation and Western Blot Analysis*

Proteins were isolated from liver samples and Western Blot analysis was performed using methods described previously (Borude et al., 2012). Briefly,

frozen liver tissue was taken out of storage in the -80°C and solubilized in RIPA buffer. 100 µg of protein was loaded onto a Bis-Tris 4-12% gel (Invitrogen) and ran for 90 minutes at 150V. Proteins were transferred to PVDF (Millipore) membrane for antibody detection. All primary and secondary antibodies used for Western Blot analysis in this study were purchased from Cell Signaling Technology (Danvers, MA) and Abcam (Cambridge, MA). OGT and OGA antibodies were a generous gift from Gerald Hart at the Johns Hopkins School of Medicine. A comprehensive list of all antibodies can be found in the table below.

| **Primary Antibody** | **Company** | **Product No.** |
|---|---|---|
| Total JNK | Cell Signaling | 3708 |
| p-JNK | Cell Signaling | 4668 |
| OGT | Antibodies were a generous gift of Gerald Hart from Johns Hopkins School of Medicine | |
| OGA | | |
| Anti-O-GlcNAc (RL2) | Abcam | ab2739 |
| Cyp2E1 | Abcam | ab151544 |
| Actin | Abcam | A2066 |
| GAPDH | Cell Signaling | 2118 |

*Real Time PCR*

RNA was isolated using TRIzol (Thermo Fisher Scientific) in accordance with the manufacturer's protocol and converted to cDNA as previously described (Wolfe, Thomas et al. 2011). Real Time PCR was run on a BioRad CFX384 (Hercules, CA) using SYBR green PCR master mix (Applied Biosystems, Foster City, CA). All target genes were normalized to 18S ribosomal RNA and compared to 0 hr samples. A comprehensive list of primers can be found in the table below.

| Primer Pair | Primer Sequence |
|---|---|
| GCLC | Forward- ATCTGCAAAGGCGGCAAC<br>Reverse- ACTCCTCTGCAGCTGGCT |
| GCLM | Forward- AGGAGCTTCGGGACTGTATCC<br>Reverse- GGGACATGGTGCATTCCAAAA |
| GR | Forward- GCCTTTACCCCGATGTATCACGCTGTG<br>Reverse- TGTGAATGCCAACCACCTTTTCCTCTTTG |

*Glutathione Assay (GSH) and APAP adduct measurements*

GSH and GSSG were measured in liver homogenates as previously described (Jaeschke and Mitchell 1990). APAP adducts were measured as described before (McGill, Lebofsky et al. 2013).

*CYP2E1 Assay*

CYP2E1 activity was measured using a p-nitrophenol assay as described before (Wu and Cederbaum 2008). Briefly, microsomes were isolated from liver tissue and activity was measured by the hydroxylation of p-nitrophenol to p-nitrocatechol by CYP2E1.

*Mass Spectrometry of APAP Metabolites*

**Materials.** The standards, acetaminophen, 3-(N-acetyl-L-cysteine-S-yl) acetaminophen, 4-acetaminophen glucuronide, acetaminophen glutathione, 4-acetaminophen sulfate and 3-cysteinylacetaminophen, and the internal standards, acetaminophen-$d_4$ and acetaminophen sulfate-$d_3$, were purchased from Toronto Research Chemicals (Toronto, Canada). Other chemicals and solvents used were obtained from standard suppliers and were of reagent or analytical grade.

**Standard and Sample Preparation.** Stock solutions of acetaminophen and its conjugated metabolites were prepared at 1 mM in 50:50 water:methanol and stored at -20°C until use. All mouse serum samples were thawed on ice. Several serum samples from untreated mouse were pooled and used as blank matrix for preparation of the calibration curve. A 20 µl aliquot of mouse serum sample was added to 90 µl of methanol (containing 200 ng/ml of acetaminophen-$d_4$ and 400 ng/ml of acetaminophen sulfate-$d_3$) to precipitate the proteins. Then, 70 µl of water and 20 µl of 50:50 water:methanol were added. Samples were vortexed for 10 sec and centrifuged for 10 min at 13,400 × g and 4°C. The standards were prepared following the same procedure but replacing the 20 µl of 50:50 water:methanol by 20 µl of working standards in 50:50 water:methanol. The calibration standards ranged from 0.5 µM to 75 µM for acetaminophen glutathione and 0.25 µM to 75 µM for all the other analytes.

**Analytical Methods.** All samples were analyzed by LC-MS/MS with a Waters ACQUITY Ultra-Performance Liquid Chromatography (UPLC®) system interfaced by electrospray ionization with a Waters Quattro Premier XE triple quadropole mass spectrometer (Waters Corp., Milford, MA) operated in positive mode with multiple reaction monitoring (MRM) scan type. The following source conditions were applied: 3.0 kV for the capillary voltage, 120°C for the source temperature, 350°C for the desolvation temperature, 50 l/hr for the source gas flow and 600 l/hr for the desolvation gas flow. The following mass transitions and collision energies (CE) were used to detect the respective analytes: 152>110, CE = 15 V

for acetaminophen; 313>208, CE = 20 V for 3-(N-acetyl-L-cysteine-S-yl) acetaminophen; 328>152, CE = 20 V for 4-acetaminophen glucuronide; 457>140, CE = 22 V for acetaminophen glutathione; 232>152, CE = 15 V for 4-acetaminophen sulfate; 271>140, CE = 24 V for 3-cysteinylacetaminophen; 156>114, CE = 15 V for acetaminophen-$d_4$ and 235>155 CE = 15 V for acetaminophen sulfate-$d_3$. The different compounds were separated on a Waters UPLC® HSS T3 column (1.8 µm, 2.1 x 150 mm) maintained at 50°C and using a gradient at 0.4 ml/min flow rate of mobile phase A (6 mM ammonium acetate in water with 0.01% formic acid) and mobile phase B (methanol) as follows: 2% B held for 0.5 min, increased to 75% over 3.5 min followed by an increase to 98% over 0.5 min and held at 98% for 2.0 min. Acetaminophen and its metabolites were quantified by back calculation of a weighted (1/x), linear least squares regression. The regression fit used the analyte/internal standard peak area ratios calculated from the calibration. Acetaminophen-$d_4$ was used as the internal standard for acetaminophen and acetaminophen sulfate-$d_3$ was used as the internal standard for all the metabolites. The MS peaks were integrated using QuanLynx software (version 4.1, Waters Corp., Milford, MA).

*Statistical Analysis*

Data presented as bar graphs show the mean ± SE. Statistical significance was calculated using Student's t-test and values were considered statistically significant if $P \leq 0.05$.

## 2.4 Results

*Increased O-GlcNAcylation in livers after APAP overdose*

Increase in total liver lysate protein O-GlcNAcylation was observed between 3 to 24 hr after APAP treatment in all molecular weight proteins as indicated by Western blotting (Fig.2.4.2B). Increased in O-GlcNAcylation correlates with increased liver injury as measured by serum ALT levels (Fig.2.4.2C). To further determine the role of O-GlcNAcylation in liver injury following APAP overdose, we generated hepatocyte specific OGT KO mice as described in the methods. The deletion of OGT was confirmed by Western Blot analysis for total liver extracts (Fig.2.4.2D). A significant decrease in OGT and a concomitant decline in total O-GlcNAcylation were observed in OGT KO mice. The small amount of OGT left is most likely in the non-parenchymal cells of the liver as TBG-Cre is only active in hepatocytes.

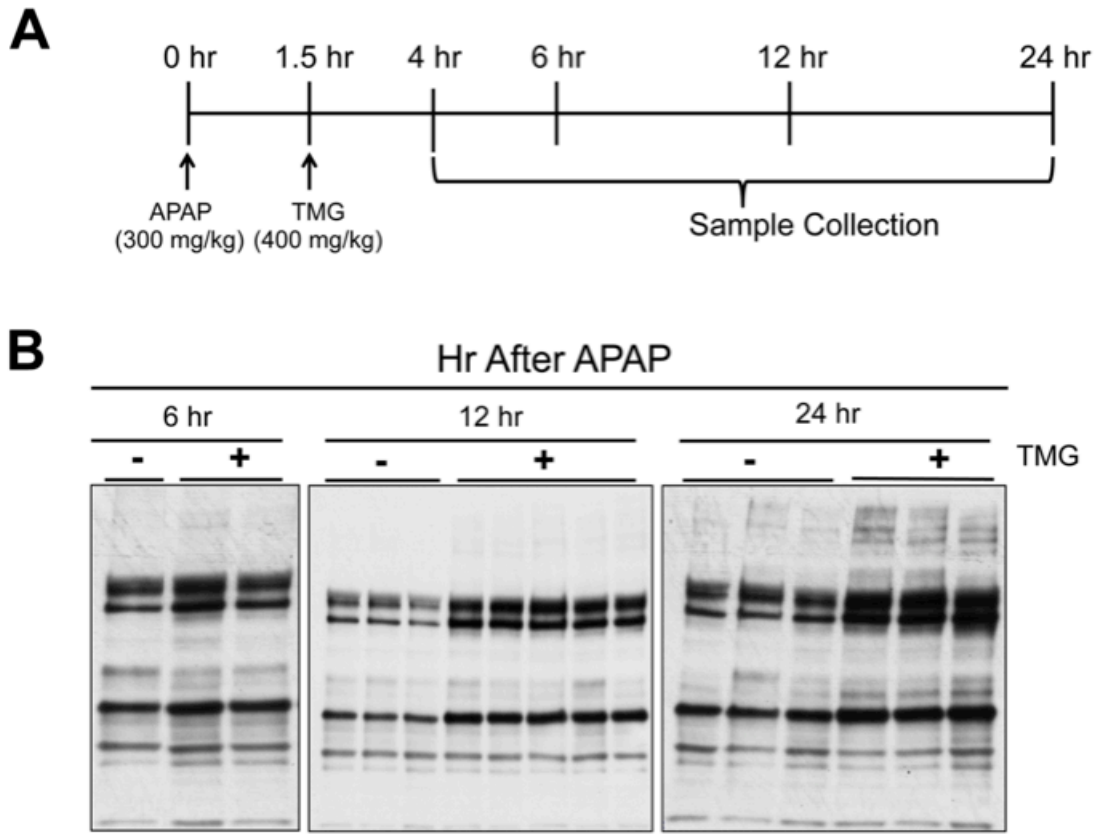



**Fig.2.4.1**. Experimental design to test the effects of increased hepatic O-GlcNAcylation on APAP toxicity. (A) Scheme showing experimental design to test the role of increased O-GlcNAcylation on APAP-induced liver injury (B) Western Blot analysis of total O-GlcNAc levels at 6, 12 and 24 hr after APAP treatment using total liver cell extracts from mice treated with APAP+saline or APAP+TMG as described in the methods.

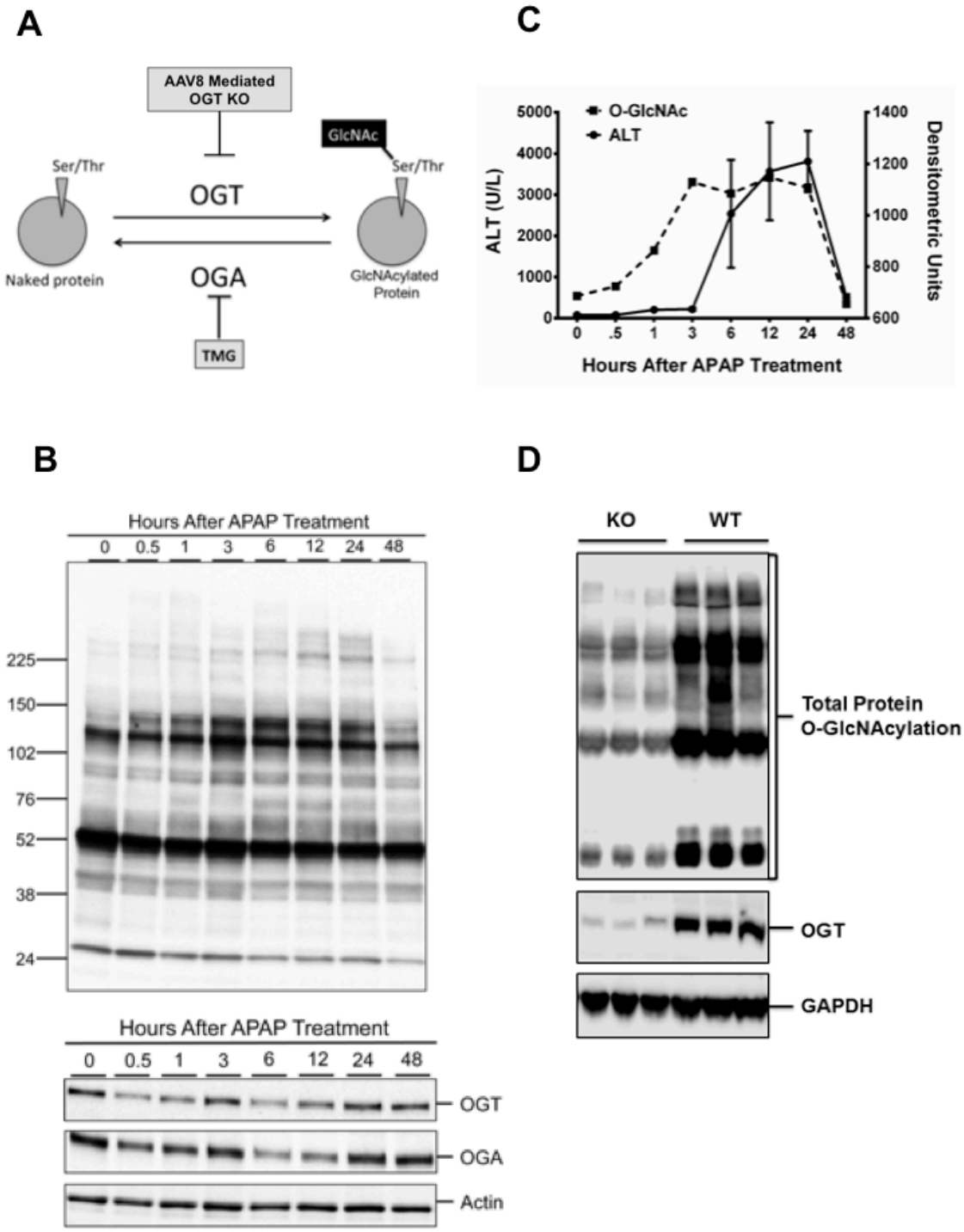

A
AAV8 Mediated
OGT KO
GlcNAc
Ser/Thr
Ser/Thr
OGT
OGA
Naked protein
GlcNAcylated
Protein
TMG
C
O-GlcNAc
ALT
ALT (U/L)
Densitometric Units
Hours After APAP Treatment
B
Hours After APAP Treatment
0 0.5 1 3 6 12 24 48
225
150
102
76
52
38
24
Hours After APAP Treatment
OGT
OGA
Actin
D
KO
WT
Total Protein
O-GlcNAcylation
OGT
GAPDH

**Fig.2.4.2.** Changes in O-GlcNAcylation after APAP overdose in mice. (A) Schematic showing O-GlcNAcylation of proteins, role of OGT and OGA, AAV8 driven deletion of OGT resulting in lower cellular O-GlcNAc, and TMG-mediated OGA inhibition resulting in increased cellular O-GlcNAc. (B) Western blot analysis of O-GlcNAcylated proteins, OGT, and OGA using total mouse liver extracts at various time points after APAP treatment (300 mg/kg) to C57BL/6 mice. (C) Line graph showing serum ALT (Left Y-axis) and densitometry analysis of hepatic O-GlcNAcylation Western blot (right Y-axis) in APAP treated mice. Densitometry was performed on total O-GlcNAc blots. The data were normalized to Actin blot densitometry. (D) Western blot analysis of OGT KO and WT mice showing a decrease in protein O-GlcNAcylation and decrease in OGT protein levels in the KO mice.

*OGT KO mice are resistant to APAP-induced acute liver injury*

WT and OGT KO mice were treated with 300 mg/kg APAP. Serum ALT levels indicated a significant decrease in liver injury at 6, 12, and 24 hr post APAP treatment in OGT KO mice compared to WT mice (Fig.2.4.3A). These findings were corroborated by histopathological analysis by H&E staining (Fig.2.4.3B). WT mice showed extensive centrilobular necrosis as expected within 6 hours after APAP exposure. The OGT KO mice exhibited a moderate change in hepatic histology but no frank cell death based on the relationship between necrosis and ALT in the early stages of APAP induced liver injury. OGT KO mice also showed depletion of glycogen upon fasting as seen in PAS stained slides, but glycogen was rapidly replenished by 1 hr post APAP treatment (Fig.2.4.4). WT mice had ablation of glycogen upon treatment with APAP at 1 hr but regained glycogen by 24 hr post APAP (Fig.2.4.4).

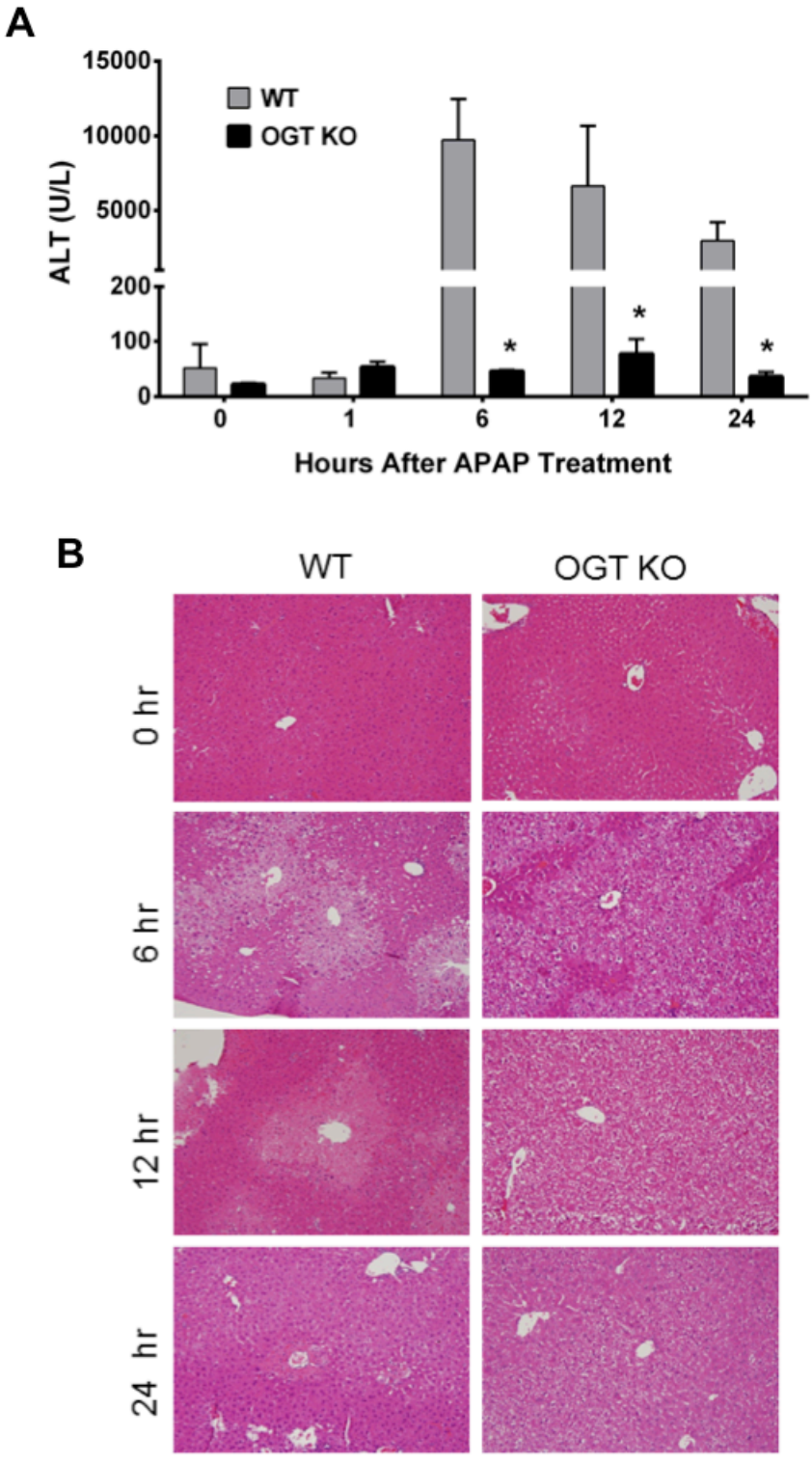

A
15000
10000
5000
200
100
0
ALT (U/L)
WT
OGT KO
*
*
*
0
1
6
12
24
Hours After APAP Treatment
B
WT
OGT KO
0 hr
6 hr
12 hr
24 hr

**Fig.2.4.3.** OGT KO mice have decreased liver injury compared to WT mice. (A) Serum ALT levels in mice treated with 300 mg/kg APAP from 0-24 hr after APAP treatment. (B) Representative photomicrographs of H&E stained liver sections from 300 mg/kg APAP treated mice at 0, 12, and 24 hr. *Indicates statistically significant difference at $P<0.05$ between the groups at the same time point.

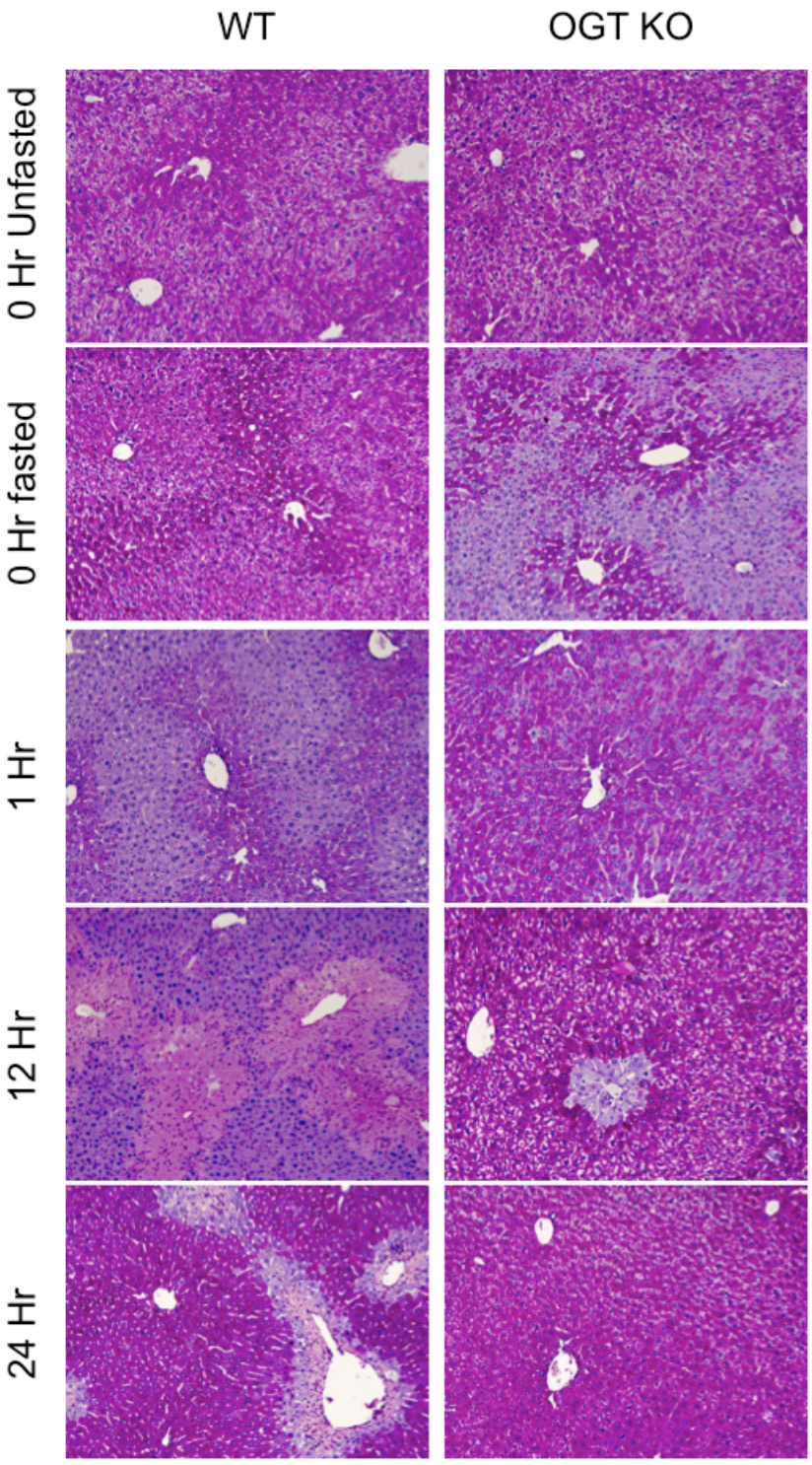



**Fig.2.4.4**. PAS stain showing OGT KO has rapid depletion of glycogen upon fasting, but rapid replenishment after APAP treatment.

*OGT KO mice did not have altered APAP metabolism*

To determine whether the lower liver injury in the OGT KO mice is due to decreased APAP metabolism we determined hepatic CYP2E1 activity, APAP metabolite levels and adduct formation. There was no difference in microsomal CYP2E1 activity as measured by p-nitrophenol assay between WT and OGT KO mice (Fig.2.4.5A). Interestingly, we observed significantly less APAP-protein adducts at 1 and 6 hr in the OGT KO compared to WT mice (Fig.2.4.5B). We further analyzed the APAP metabolite profile using LC-MS, which showed that OGT KO and WT had similar levels of APAP-glucuronide and APAP-sulfate at 1 hr, but those levels were significantly decreased in OGT KO mice at 6 and 12 hr (Fig.2.4.5C-D). Studies have shown that hepatic JNK activation is a critical and indispensable step in APAP-induced liver injury (Du, Xie et al. 2015). Western Blot analysis showed no increase in activation of JNK by phosphorylation (p-JNK) in OGT KO mice, but significant increase in JNK activation in WT mice (Fig.2.4.5E).

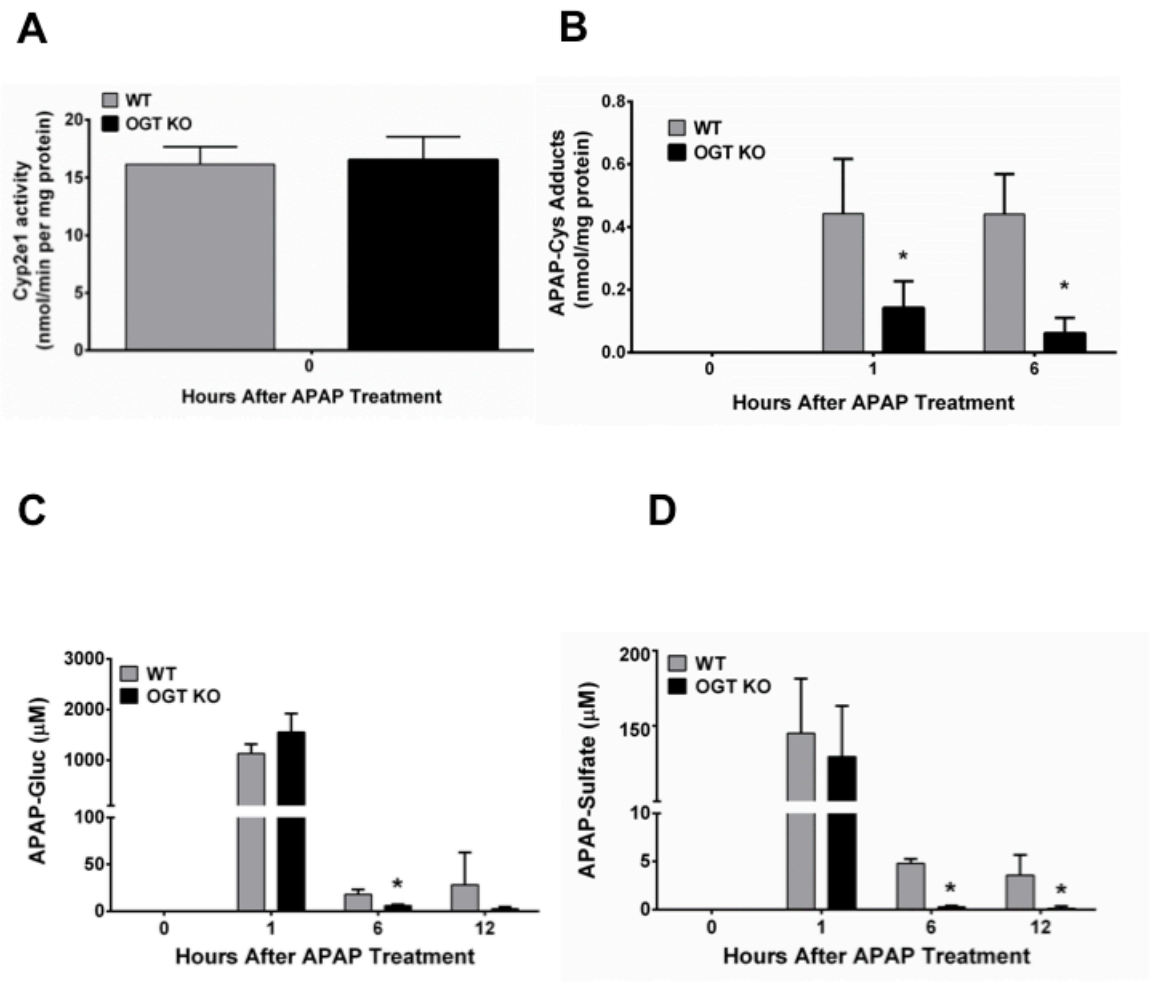

A
B
WT
OGT KO
Cyp2e1 activity (nmol/min per mg protein)
Hours After APAP Treatment
APAP-Cys Adducts (nmol/mg protein)
C
D
APAP-Gluc (μM)
APAP-Sulfate (μM)
E
0 Hr
1 Hr
6 Hr
12 Hr
24 Hr
WT KO
p-JNK
Total JNK
GAPDH

**Fig.2.4.5.** OGT KO mice have no alteration in CYP2E1 levels but have changes in APAP metabolite formation. (A) p-Nitrophenol assay for CYP2E1 activity from isolated liver microsomes. (B) APAP-Cys protein adduct formation in whole liver lysate in mice treated with 300 mg/kg APAP at 0, 1, and 6 hr. LC-MS data showing (C) APAP-Glucuronide, and (D) APAP-Sulfate. (E) Western Blot of total and phosphorylated JNK activation using total liver extracts of mice treated with 300 mg/kg APAP from 0-24 hr after APAP treatment. *Indicates statistically significant difference at $P<0.05$ between the groups at the same time point.

*OGT KO mice had rapid replenishment of hepatic glutathione*

GSH is the main cellular antioxidant molecule that quenches NAPQI, the reactive metabolite of APAP, and scavenges mitochondrial ROS and peroxynitrite. We determined GSH levels over a time course of 0 to 24 hr. Hepatic GSH levels were similar in OGT KO and WT mice at 0 hr (Fig.2.4.6A). Further, both groups had similar depletion of GSH at 1 hr after APAP treatment. However, the OGT KO mice had significantly higher and faster replenishment of GSH levels as compared to WT mice. The OGT KO mice regained baseline GSH level within 6 hr after APAP treatment but the WT mice did not regain baseline GSH levels until 24 hr post APAP treatment (Fig.2.4.6A). To determine whether rapid GSH replenishment in OGT KO mice is due to induction of genes in the GSH biosynthesis pathway, we performed Real Time PCR for Glutamate-Cysteine ligase regulatory (GCLM) and catalytic (GCLC) subunits. The data indicate that both GCLM and GCLC were significantly increased in OGT KO mice compared to WT mice 1 and 6 hr (Fig.2.4.6B-C). Furthermore, Glutathione reductase (GR), an enzyme important for the recycling of GSH from the disulfide (GSSG) form, was also significantly increased in OGT KO mice at 1 and 6 hr when compared to WT mice (Fig.2.4.6D).

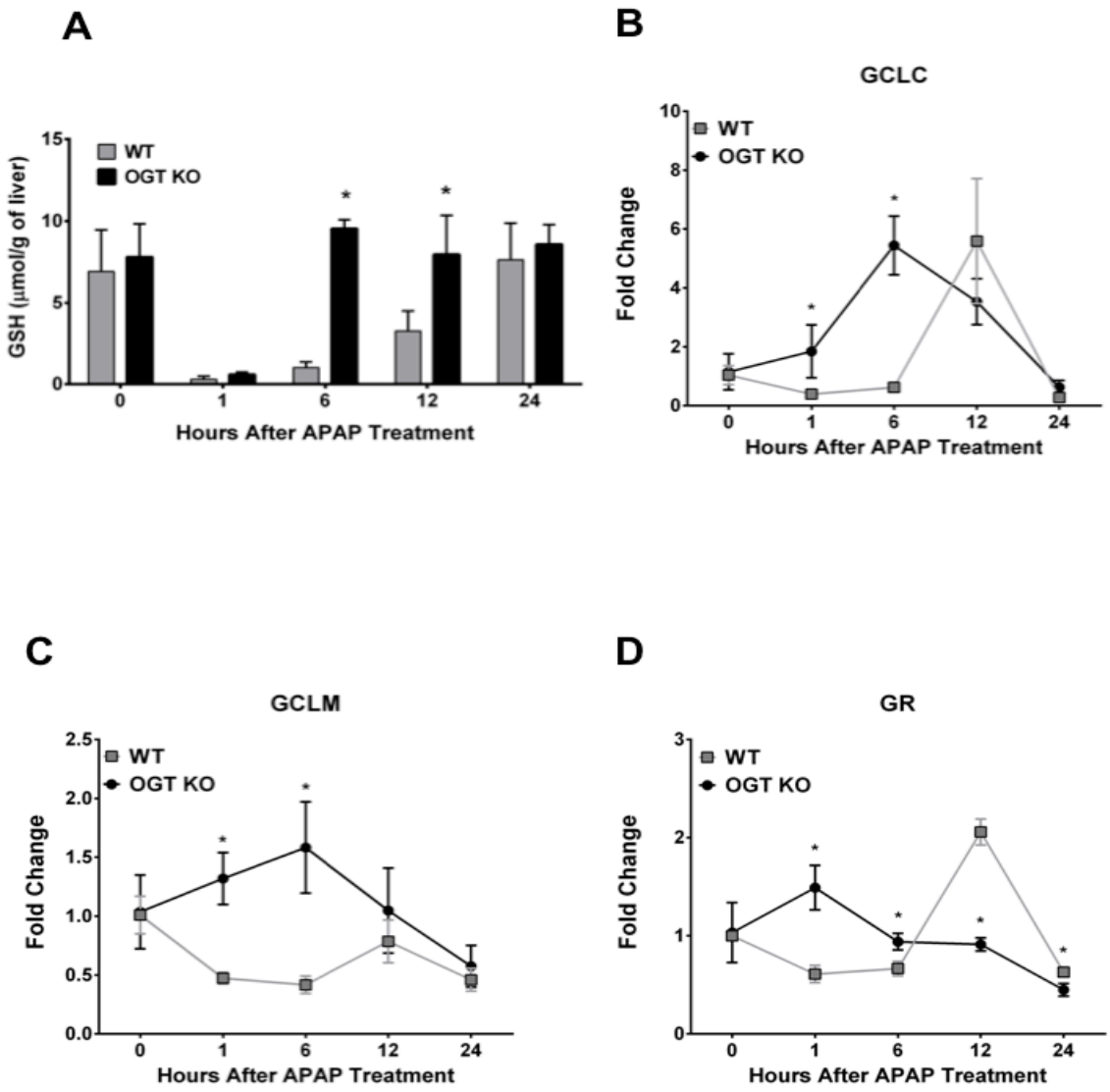



**Fig.2.4.6.** OGT KO have rapid replenishment of GSH after APAP overdose (A) Hepatic GSH levels and real time PCR of GSH metabolism enzymes (B) GCLC, (C) GCLM, and (D) GR in mice treated with 300 mg/kg APAP at 0, 1, 6, 12, 24 hr.

*TMG increases O-GlcNAc levels in livers of APAP treated mice*

Next, we determined the effect of increased O-GlcNAcylation on APAP toxicity. Preliminary dose finding studies indicate that 400 mg/kg TMG was a suitable dose to induce enhanced hepatic O-GlcNAcylation within 18 hr (data not shown). Western blotting indicted that Male C57BL/6J mice treated with 400 mg/kg dose of TMG 1.5 hr after the administration of a 300 mg/kg dose of APAP (Fig2.4.1A) showed increased liver O-GlcNAcylation as compared to mice treated with saline (Fig.2.4.1B). A moderate increase in O-GlcNAcylation was observed at 6 hr, which further increased at 12 and 24 hr after APAP treatment (Fig2.4.1B).

*Increased APAP-induced liver injury in mice with higher O-GlcNAc*

Serum ALT levels and histopathological analysis of H&E stained liver sections showed significantly higher APAP-induced liver injury following acute TMG treatment (Fig.2.4.7A-B). Serum ALT was significantly increased following chronic TMG treatment at 24 hr (Fig2.4.8A). Both serum ALT activities (Fig.2.4.7A) and the areas of necrosis (Fig.2.4.7B-C) were significantly higher in acute TMG-treated mice with increased O-GlcNAc levels at 6 and 12 hr post-APAP. Liver injury decreased 24 hr after APAP treatment in both control mice and in mice with increased O-GlcNAc levels as demonstrated by the decrease in serum ALT levels and improved histology.

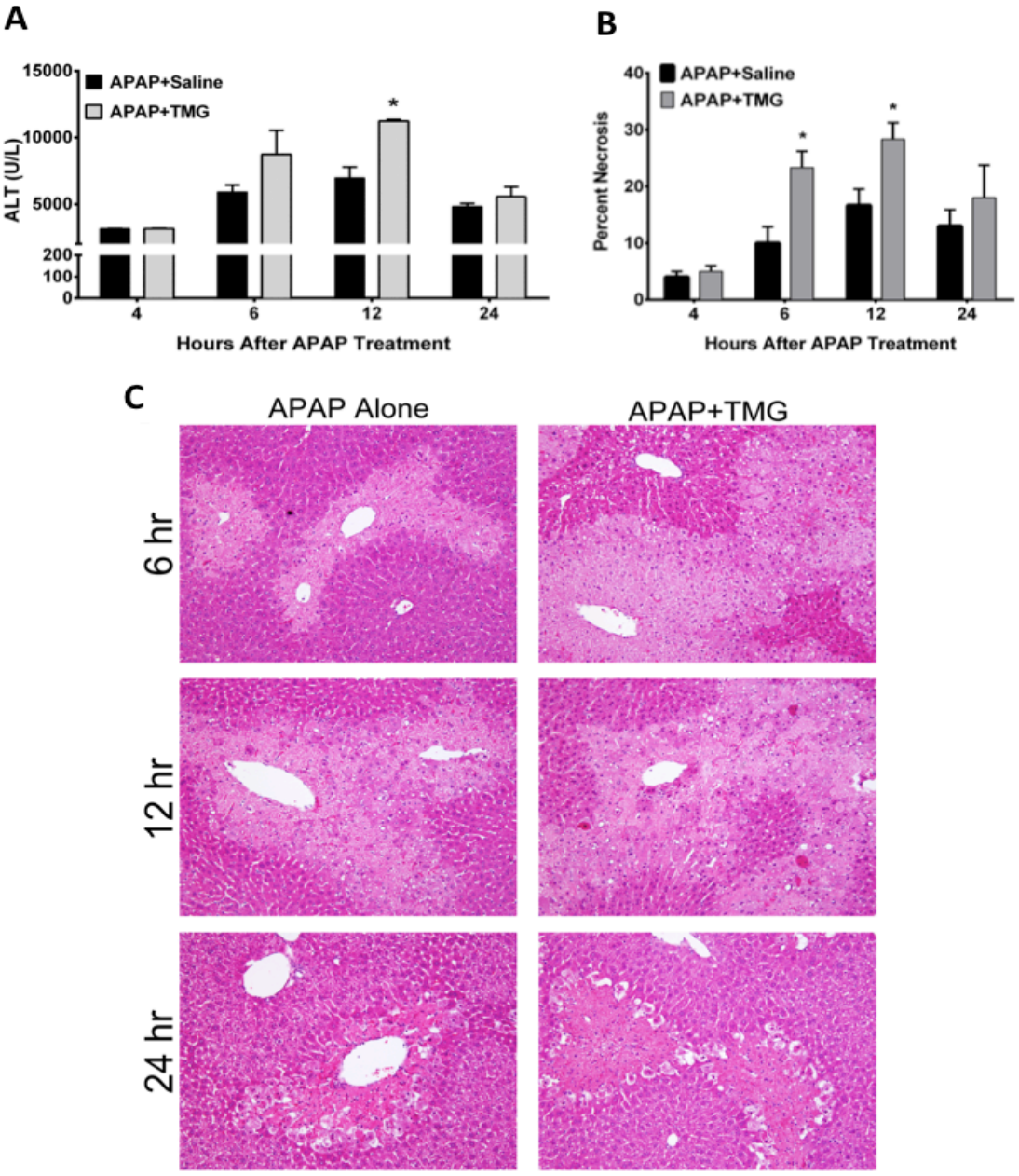

A
15000
10000
5000
200
100
0
ALT (U/L)
APAP+Saline
APAP+TMG
*
4
6
12
24
Hours After APAP Treatment
B
40
30
20
10
0
Percent Necrosis
APAP+Saline
APAP+TMG
*
*
4
6
12
24
Hours After APAP Treatment
C
APAP Alone
APAP+TMG
6 hr
12 hr
24 hr

**Fig.2.4.7.** Increased liver injury in mice with acute increased O-GlcNAc. (A) Serum ALT levels in mice treated with APAP+saline and APAP+TMG at 4, 6, 12 and 24 hr after APAP administration. *Indicates statistically significant difference at $P<0.05$ between the groups at the same time point. (B) Percent necrosis in mice treated with APAP+saline and APAP+400 mg/kg TMG at 4, 6, 12 and 24 hr after APAP administration. (C) Representative photomicrographs of H&E stained liver sections from APAP+saline and APAP+400 mg/kg TMG treated mice at 6, 12, and 24 hr after APAP dose.

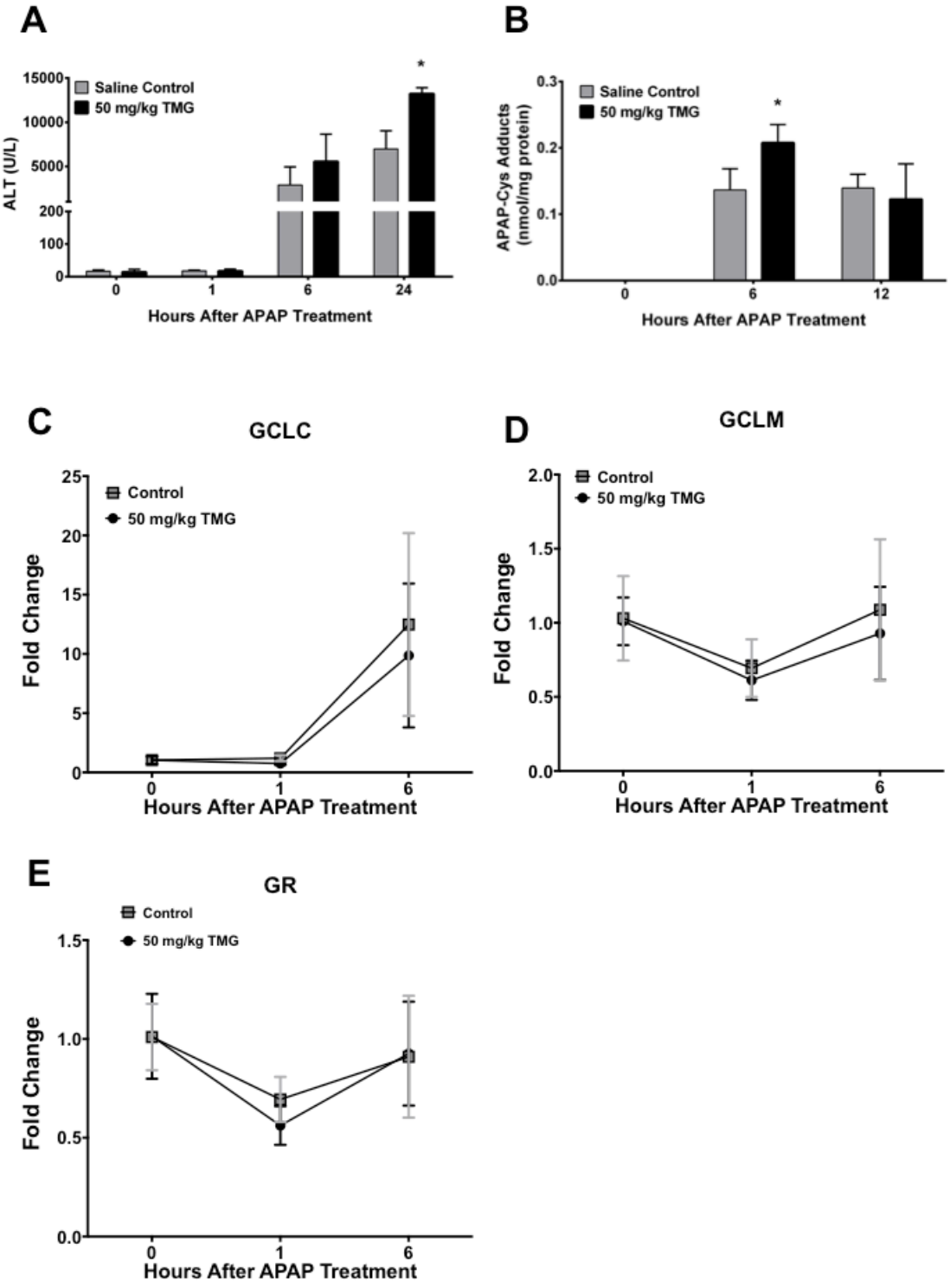
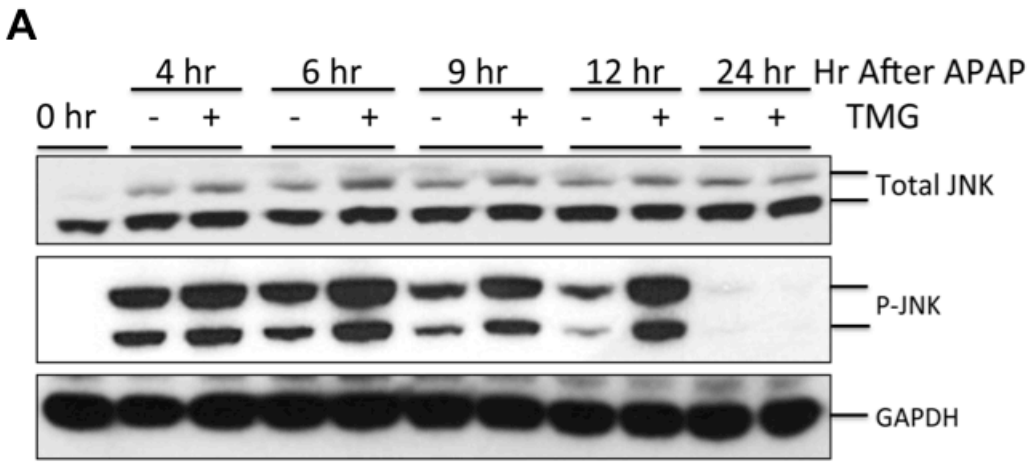

A
Saline Control
50 mg/kg TMG
ALT (U/L)
15000
10000
5000
200
100
0
*
0
1
6
24
Hours After APAP Treatment
B
Saline Control
50 mg/kg TMG
APAP-Cys Adducts (nmol/mg protein)
0.3
0.2
0.1
0.0
*
0
6
12
Hours After APAP Treatment
C
GCLC
Control
50 mg/kg TMG
Fold Change
25
20
15
10
5
0
0
1
6
Hours After APAP Treatment
D
GCLM
Control
50 mg/kg TMG
Fold Change
2.0
1.5
1.0
0.5
0.0
0
1
6
Hours After APAP Treatment
E
GR
Control
50 mg/kg TMG
Fold Change
1.5
1.0
0.5
0.0
0
1
6
Hours After APAP Treatment

**Fig.2.4.8.** Increased liver injury in mice with chronic increased O-GlcNAc. (A) Serum ALT levels in mice treated with and 50 mg/kg TMG for 2 weeks followed by 300 mg/kg APAP and 300mg/kg APAP only at 1, 6 and 24 hr after APAP administration. (B) APAP-Cys protein adduct formation in whole liver lysate in mice treated with 300 mg/kg APAP at 0, 1, and 6 hr *Indicates statistically significant difference at $P<0.05$ between the groups at the same time point. Real time PCR of GSH metabolism enzymes (C) GCLC, (D) GLCM, and (E) GR in mice treated 50 mg/kg TMG for 2 weeks followed by 300 mg/kg APAP and 300mg/kg APAP only at 0, 1, and 6 hr.

*Sustained increase in hepatic JNK activation in mice with higher O-GlcNAc after APAP treatment*

We performed further analysis to determine the mechanisms of increased APAP-induced liver injury in mice with higher O-GlcNAc levels. Western blot analyses revealed higher JNK activation (increased phosphorylation of JNK) in acute TMG-treated mice as compared to control mice at 6 and 12 hr post-APAP administration. In control mice JNK activation peaked at 4 hr and declined thereafter. However, in acute TMG-treated mice, JNK activation was prolonged at least until 12 hr post-APAP treatment (Fig.2.4.9A-B). Further analysis revealed higher mitochondrial JNK activation in mice with higher O-GlcNAc at 9 hr post APAP treatment (data not shown).


A
4 hr
6 hr
9 hr
12 hr
24 hr
Hr After APAP
0 hr
- + - + - + - + - +
TMG
Total JNK
P-JNK
GAPDH

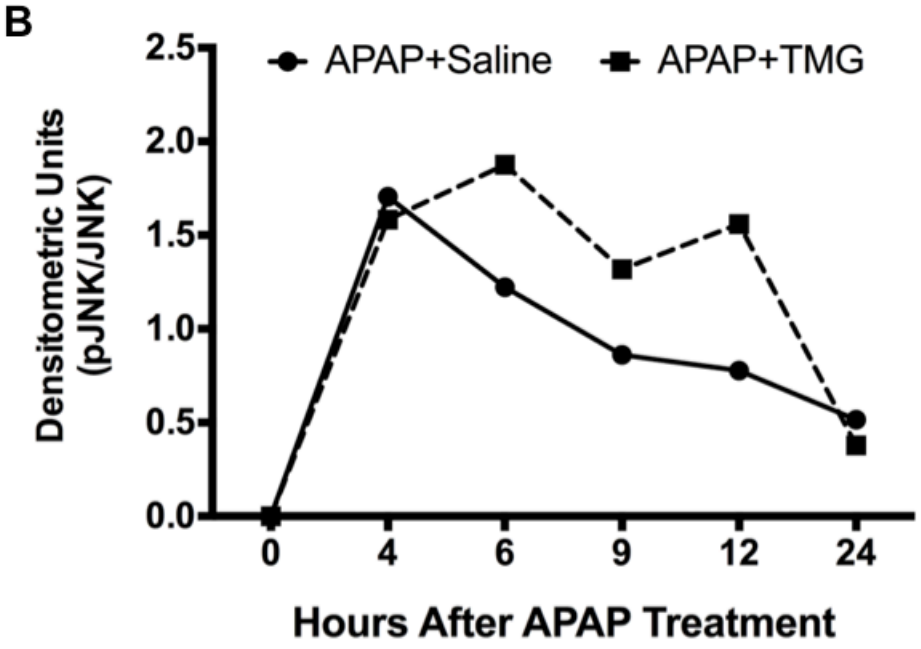

B
APAP+Saline
APAP+TMG
Densitometric Units
(pJNK/JNK)
2.5
2.0
1.5
1.0
0.5
0.0
0
4
6
9
12
24
Hours After APAP Treatment

**Fig.2.4.9.** Increased and sustained JNK activation after acute TMG treatment. (A) Western Blot and (B) Densitometry of the blots of total and phosphorylated JNK activation using total liver extracts of mice treated with APAP+saline and APAP+400 mg/kg TMG at 0, 4, 6, 9, 12 and 24 hr after APAP treatment.

*No Change in APAP metabolism after acute TMG treatment*

To determine whether the increase in APAP-induced liver injury observed in mice with higher O-GlcNAc is due to increased APAP metabolism, we measured three factors indicative of APAP metabolism including hepatic CYP2E1 levels, GSH depletion and APAP adduct formation. Western blot analysis of total liver lysates obtained from mice treated with acute TMG only at 400 and 800 mg/kg indicated no change in CYP2E1 expression (Fig.2.4.10A). HPLC-based analysis of APAP-protein adducts showed an equal degree of protein adduct formation in control mice and in mice with higher O-GlcNAcylation due to acute TMG treatment after APAP treatment (Fig.2.4.10B). Chronic treatment of TMG resulted in increase in APAP-protein adduct formation at 6 hr (Fig.2.4.8B). Analysis of hepatic GSH content revealed significant and similar GSH depletion in both control and acute TMG-treated mice following APAP treatment. Whereas there was no difference in hepatic GSH content or depletion of GSH post APAP treatment, a moderate delay in the recovery of hepatic GSH after APAP treatment was observed in acute TMG treated mice between 6 and 12 hr (Fig.2.4.10C). Consistent with these data, Real time PCR analysis showed significant reduction in transcripts of GCLC and GR at 12 hr in acute TMG treated mice (Fig.2.4.10D-F). GCLM transcript levels were moderately lower but the difference was not statistically significant (Fig.2.4.10E). There was no significant difference in GCLC, GCLM, or GR in chronic TMG treated mice, but there was a trend towards an increase in GCLC and GCLM (Fig.2.4.8C-E).

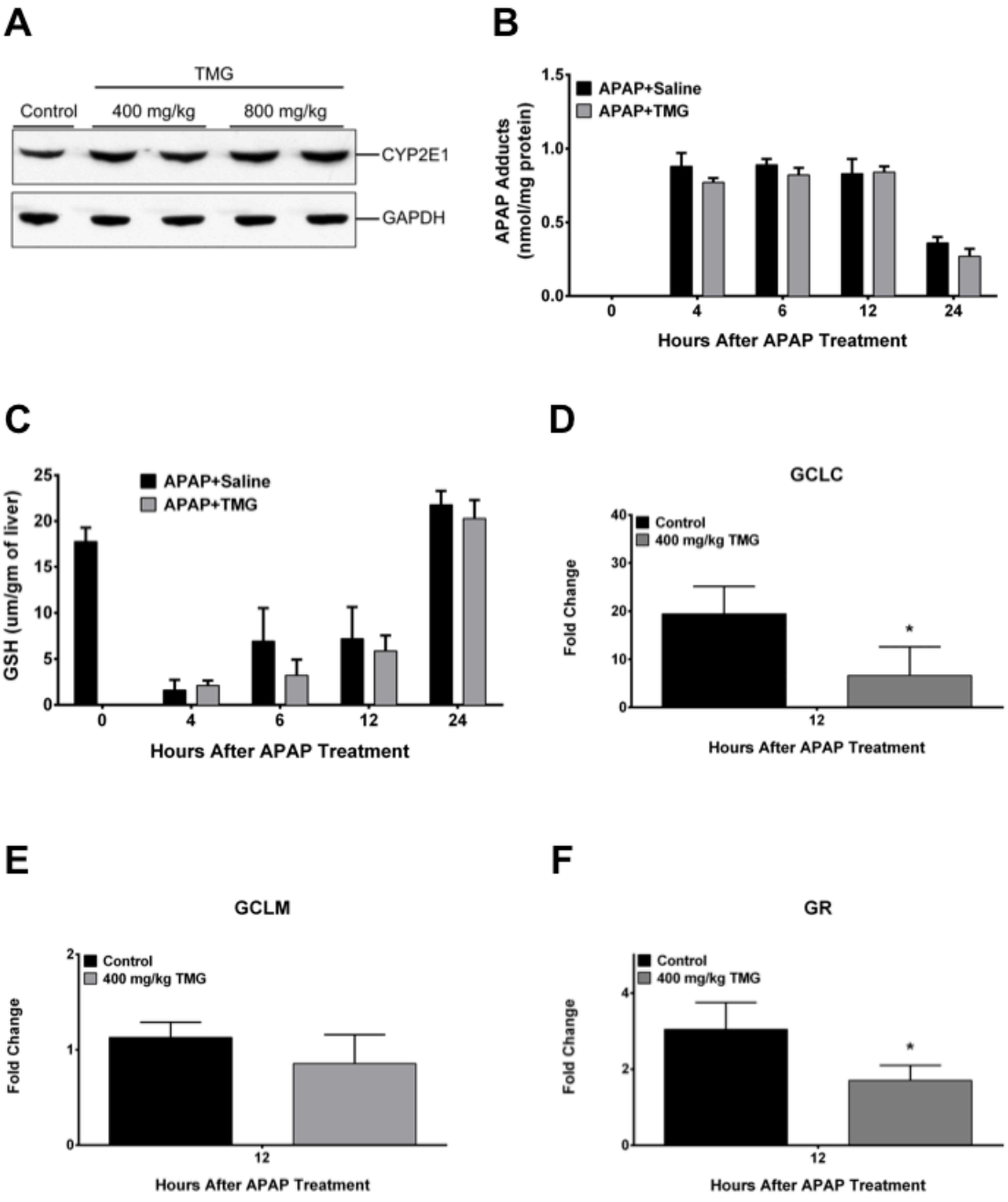

A
TMG
Control
400 mg/kg
800 mg/kg
CYP2E1
GAPDH
B
APAP+Saline
APAP+TMG
APAP Adducts (nmol/mg protein)
1.5
1.0
0.5
0.0
0
4
6
12
24
Hours After APAP Treatment
C
APAP+Saline
APAP+TMG
GSH (um/gm of liver)
25
20
15
10
5
0
0
4
6
12
24
Hours After APAP Treatment
D
GCLC
Control
400 mg/kg TMG
Fold Change
40
30
20
10
0
*
12
Hours After APAP Treatment
E
GCLM
Control
400 mg/kg TMG
Fold Change
2
1
0
12
Hours After APAP Treatment
F
GR
Control
400 mg/kg TMG
Fold Change
4
2
0
*
12
Hours After APAP Treatment

**Fig.2.4.10.** (A) Western Blot analysis for CYP2E1 using total cell extracts from mice treated with 400 or 800 mg/kg TMG dose. (B) APAP protein adducts (C) Hepatic GSH levels and in the liver in control mice, mice treated with APAP+saline and APAP+400 mg/kg TMG at 0, 4, 6, 12, and 24 hr after APAP treatments. Real time PCR of GSH metabolism enzymes (D) GCLC, (E) GLCM, and (F) GR in mice treated with APAP+400 mg/kg TMG at 12 hr. *Indicates statistically significant difference at $P<0.05$ between the groups at the same time point.

## 2.5 Discussion

Approximately 50% of all the ALF cases in many western countries are associated with overdose of APAP (Lee 1993, Lee 2003, Lee and Seremba 2008). Despite decades of research, the mechanisms of APAP-induced ALF are not completely clear (Jaeschke et al., 2012). Hence the treatment options for APAP-induced ALF remain extremely limited (Larson, Polson et al. 2005, Chung, Kim et al. 2006). Recent studies have demonstrated that post-translational modification of proteins including sumoylation, lysine conjugation, methylation, ubiquitination and O-GlcNAcylation play a central role in numerous pathophysiologic processes (Schrattenholz, Soskic et al. 2010, Osna, Carter et al. 2016, Baker, De Nardo et al. 2017, Martinez, Dias et al. 2017). Importantly, it was demonstrated that SIRT3-dependent protein deacetylation enhanced APAP-induced liver injury (Lu, Bourdi et al. 2011). However, the role of many other PTMs in APAP-induced liver injury is currently not known. Here, we demonstrate that hepatic O-GlcNAcylation plays a crucial role in APAP hepatotoxicity. Our data indicate that increase in hepatic O-GlcNAcylation of proteins results in increased APAP-induced liver injury and decreased O-GlcNAcylation inhibits APAP-induced liver injury.

To examine the effects of a decrease in O-GlcNAcylation on APAP-induced liver injury we deleted OGT, the sole enzyme involved in O-GlcNAcylation of proteins, specifically in hepatocytes, which resulted in a reduction in hepatic O-GlcNAcylation. Our studies showed that OGT KO mice displayed a significant reduction in liver injury when compared to WT mice.

Consistently, when we increased hepatic O-GlcNAcylation by TMG-mediated OGA inhibition, APAP hepatotoxicity was exacerbated. These data suggest that O-GlcNAcylation plays a critical role in initiation of APAP-induced liver injury.

It is known that APAP-induced liver injury involves a cascade of events following bio-activation of APAP to NAPQI including increased formation of reactive oxygen and reactive nitrogen species, JNK activation, mitochondrial damage, and ultimately cellular necrosis (Jaeschke and Bajt 2006, Jaeschke, McGill et al. 2012). JNK is activated by phosphorylation at Thr183/Tyr185 by upstream MAPK cascade (Seki, Brenner et al. 2012). Previous studies have shown that activation (phosphorylation) of JNK is a critical step in APAP-induced liver injury (Gunawan, Liu et al. 2006, Henderson, Pollock et al. 2007, Han, Shinohara et al. 2010, McGill, Williams et al. 2012). Our studies revealed that increasing hepatic O-GlcNAcylation after APAP overdose results in increased and prolonged total and mitochondrial JNK activation, which correlates with higher hepatic injury. Studies indicate that O-GlcNAcylation of proteins can both decrease and increase protein activity (Slawson and Hart 2011). It is possible that O-GlcNAcylation further induces hepatic JNK activation following APAP overdose and contributes to higher injury. Alternatively, O-GlcNAcylation could inhibit phosphatases that have been shown to reduce JNK activation (Wancket, Meng et al. 2012, Mobasher, Gonzalez-Rodriguez et al. 2013).

CYP2E1 is the most important cytochrome P450 enzyme responsible for phase two metabolism of APAP (McGill and Jaeschke 2013). Our data showed that there was no difference in hepatic CYP2E1 expression (data not shown) or

activity following OGT deletion. Further, no difference in basal GSH levels and the extent of GSH depletion after APAP treatment in WT and OGT KO mice was observed. However, APAP-protein adducts were decreased by approximately 50% in the OGT KO mice. The decrease in quantity of protein adducts in spite of equal CYP2E1 activity and basal GSH levels indicate that O-GlcNAcylated proteins may have higher susceptibility to forming APAP-protein adducts. These data are consistent with previous observations that protein modifications such as acetylation of lysine residues attenuated protein adduct formation and protected against APAP hepatotoxicity. Other major mechanisms behind the protection observed in OGT KO mice were significantly faster GSH replenishment in OGT KO mice following APAP treatment. APAP-induced cell death is critically dependent on the mitochondrial oxidant stress and peroxynitrite formation after initial GSH depletion and protein adduct formation (Du, Ramachandran et al. 2016). The lack of GSH during this initial phase substantially impairs the antioxidant defense of the cell. Consequently, it was shown that providing substrates for GSH synthesis (N-acetylcysteine, GSH) during this time accelerates the GSH recovery in the cell, and most importantly in the mitochondria, protecting against APAP-induced liver injury (Knight, Ho et al. 2002, James, McCullough et al. 2003, Saito, Zwingmann et al. 2010). Moreover, female mice demonstrate a more pronounced induction of GCLC after APAP compared to male mice and the resulting faster recovery of hepatic GSH levels makes these female mice much less susceptible to APAP hepatotoxicity than male mice (Du, Williams et al. 2014). On the other hand, overexpression of

GCLC or GCLM in male mice also renders them less susceptible to APAP-induced liver injury (Botta, Shi et al. 2006, Botta, White et al. 2008). Together, these data demonstrate the importance of the hepatic GSH synthesis capacity as a critical determinant of the susceptibility to APAP-induced hepatotoxicity. To examine the mechanism behind the rapid replenishment of GSH following OGT deletion, we examined expression of the key enzymes in GSH metabolism. The data indicate a significant up regulation of three key enzymes in GSH metabolism including GCLM, GCLC and GR in the OGT KO mice following APAP treatment. These findings explain the faster recovery of hepatic GSH levels and the resistance to APAP hepatotoxicity. It is known that the enzymes involved in GSH synthesis are regulated by the nuclear receptor NRF2 (Wild and Mulcahy 2000, Enomoto, Itoh et al. 2001), which plays a critical role in protection against oxidative stress in the liver. Interestingly, recently published data (Chen, Smith et al. 2017) and studies from our group have shown that NRF2 is O-GlcNAcylated, and the treatment of mice with TMG leads to a loss of NRF2 expression while OGT KO animals have an increase in NRF2 expression (Tan, McGreal et al. 2017). Our data are consistent with this observation and suggest that deletion of OGT may reduce O-GlcNAcylation of NRF2 resulting in increased protein levels. Stabilization of NRF2 may lead to a rapid induction in GSH biosynthesis genes, faster replenishment of GSH and restricting progression of APAP-induced liver injury. In the opposite condition, expression of GSH biosynthetic genes is down regulated in TMG-treated mice, which have higher O-GlcNAcylation. Taken together, these data indicate that NRF2 activity is regulated by O-GlcNAcylation,

which in turn regulates GSH replenishment and the extent of APAP-induced liver injury.

Collectively, The data presented in this study are significant because chemicals that increase O-GlcNAcylation, and specifically TMG, are being tested as therapeutic agents for neurological disorders including Alzheimer's disease (Macauley and Vocadlo 2010). Another issue is potential interaction of APAP with the dietary supplement glucosamine. The availability of O-GlcNAc is controlled by flux through the hexosamine biosynthetic pathway (HBP) (Teo, Wollaston-Hayden et al. 2010). Glucose enters this pathway and is converted to UDP-GlcNAc, the metabolite for OGT. Glucosamine feeds directly into the HBP, skipping the initial step, and can result in increased O-GlcNAcylation. This is important because glucosamine is a popular dietary supplement used for its perceived benefits for arthritis and joint pain (Miller and Clegg 2011). The addition of glucosamine to a pain regimen that already includes APAP could potentially be hazardous. Our data suggests that any therapeutic interventions that increase O-GlcNAcylation should be carefully tested for its safety, drug-drug interactions and possible hepatotoxic effects; while methods to decrease cellular O-GlcNAc might be promising new therapeutics targets for the treatment of APAP overdose and APAP-induced liver injury.

In conclusion, our data demonstrate that decreasing hepatic O-GlcNAcylation, a ubiquitous PTM of key proteins, drastically reduced the toxic effects of APAP by a dual effect. First, reduced O-GlcNAcylation attenuates targets for protein adduct formation and second, the promotion of GSH recovery

enhances the scavenging capacity for ROS and peroxynitrite in mitochondria. These studies have revealed a novel pathway that critically affects not only APAP-induced liver injury but also highlighted a possible causal role of O-GlcNAcylation in drug- and chemical-induced liver injury where oxidative stress is a major mechanism.

# Chapter 3- Decreased O-GlcNAcylation by hepatocyte specific deletion of OGT mice results in enhanced liver regeneration

## 3.1 Abstract

O-GlcNAcylation is the covalent attachment of a single sugar N-acetyl-glucosamine (O-GlcNAc) sugar to a Ser/Thr residue of a protein. O-GlcNAc transferase (OGT) is the enzyme that adds O-GlcNAc moieties to proteins, While O-GlcNAcase (OGA) removes O-GlcNAc moieties from proteins. O-GlcNAcylation has extensive crosstalk with protein phosphorylation and because of this it plays a critical role in cell signaling involved in cell proliferation and differentiation. However, the role of O-GlcNAc in liver regeneration is not known. In this study, we examine the role of O-GlcNAc in liver regeneration after partial hepatectomy (PHX) following hepatocyte specific deletion of OGT in mice. WT mice had a normal regenerative response with peak proliferation at 48 hr after PHX, which subsided by 7 days and regeneration was complete at 14 days post PHX. OGT KO mice had a similar profile of cell proliferation up to 48 hr but exhibited significantly higher cell proliferation at 5, 7, and 14 days as compared to WT mice. Even at 14 days post PHX, substantial number of cells were proliferating in the OGT KO mice, which resulted in significantly higher liver to body weight ratio. The increased proliferation was secondary to an increase in Cyclin D1, pRb and CDK4 protein levels in later time points when compared to WT mice. Several signaling pathways critical for hepatocyte proliferation and known to be involved in cancer pathogenesis remained active in OGT KO mice significantly longer than in WT mice. Taken together, these data indicate that hepatic O-GlcNAcylation plays a critical role in regulation of cell cycle after PHX, loss of which results in termination of liver regeneration.

## 3.2 Introduction

O-linked β-N-acetyl glucosamine (O-GlcNAc) is a single sugar posttranslational protein modification covalently attached to a hydroxyl group of Ser or Thr residue of a nuclear, cytoplasmic or mitochondrial protein (Zachara, O'Donnell et al. 2004, Slawson, Copeland et al. 2010). O-GlcNAc is added to a protein by a single enzyme, O-GlcNAc transferase (OGT), in a process called O-GlcNAcylation. In the reverse reaction, a single enzyme called O-GlcNAcase (OGA) performs the removal of O-GlcNAc from a protein. The cellular source of O-GlcNAc is the flux of 2-3% of the glucose intake through the hexosamine biosynthetic pathway (HPB) to form the high-energy donor substrate UDP-GlcNAc (Slawson, Copeland et al. 2010, Bond and Hanover 2015). HBP is at the intersection of several other key pathways including the metabolism of carbohydrates, fats, amino acids, and nucleotides; this makes HBP flux, and thus O-GlcNAcylation, very sensitive to metabolite availability and nutrient flux (Bond and Hanover 2015). O-GlcNAcylation has been shown to have extensive crosstalk with protein phosphorylation (Slawson and Hart 2003). When O-GlcNAc is elevated in mammalian cells, 70% of phosphorylation sites are affected, making it extremely important in signaling cascades and enzyme function (Wang, Gucek et al. 2008).

O-GlcNAcylation is shown to regulate many cellular processes including cell growth and cell survival (Slawson and Hart 2011). O-GlcNAc also plays a pivotal role in the cell cycle and cell cycle progression (Slawson, Zachara et al. 2005). Elevated O-GlcNAc is shown to be a key regulator of mitotic

phosphorylation and overexpression of either OGT or OGA disrupts cyclin periodicity leading to improper cytokinesis and cell growth delays in many cell types (Slawson, Zachara et al. 2005). A deletion of OGT in mouse embryonic fibroblasts leads to an increased expression of the cyclin inhibitor p27 and growth delays (Zachara, O'Donnell et al. 2004). The reduction of OGA leading to an increase in O-GlcNAc was shown to promote mitotic spindle formation defects (Tan, Caro et al. 2013, Lanza, Tan et al. 2016), and a knockdown of OGA in HeLa cells caused a prolonged M phase with shortened spindle chromatids and increased multi-pole spindles (Lanza, Tan et al. 2016). The process of O-GlcNAcylation is tightly regulated and any alteration is O-GlcNAc cycling has a profound effect on the cell cycle.

The liver has an innate ability to regenerate, which plays a critical role in maintaining liver homeostasis (Michalopoulos 2017). Liver regeneration after partial hepatectomy (PHX), the most commonly used model of liver regeneration, is regulated by precise and orderly signaling events. During Liver regeneration, first the hepatocytes followed by other hepatic cells in the remnant liver lobes undergo cellular proliferation to restore the liver mass (Michalopoulos 2007). After PHX, the liver regains its pre-hepatectomy mass and function within 7 to 14 days in rodents (Apte, Gkretsi et al. 2009).

Due to the role O-GlcNAc in cell cycle progression and cyclin expression we set out to examine the role of O-GlcNAc in liver regeneration using partial hepatectomy (PHX) as a model and hepatocyte specific OGT knockout (OGT

KO) mice. Our results have revealed a novel role of this ubiquitous posttranslational modification (PTM) in the termination of liver regeneration.

## 3.3 Materials and Methods

*Animal and Tissue Preparation*

Animal studies and procedures were approved and performed in accordance with the guidelines set forth by the Institution of Animal Care and Use Committee at the University of Kansas Medical Center. OGT- floxed ($OGT^F$) mice (Shafi, Iyer et al. 2000) were provided by Natasha Zachara from the Johns Hopkins University School of Medicine. 2-3 month old male $OGT^F$ mice (n=3-5 per group per time point) were injected i.p. with AAV8.TBG.PI.Cre.rBG (Penn Vector Core, Philadelphia, PA) at $5E^{11}$ virus particles per mouse to initiate Cre-mediated deletion of OGT. 10 days after deletion, partial hepatectomy (PHX) was performed as previously described (Apte, Gkretsi et al. 2009). OGT deletion was confirmed in all OGT KO mice after sample collection using Western blot. Surgeries occurred between 9 AM and 11 AM. Mice were euthanized by cervical dislocation under isoflorane over a timecourse from 0 day – 14 days. Livers were harvested and embedded in paraffin.

*Western Blot Analysis*

Western blot analysis of isolated proteins was performed as previously described (Borude, Edwards et al. 2012). Briefly, frozen liver tissue was taken out of storage in the -80°C and solubilized in RIPA buffer. 100 µg of protein was loaded onto a Bis-Tris 4-12% gel (Invitrogen) and ran for 90 minutes at 150V. Proteins were transferred to PVDF (Millipore) and stained with primary antibodies purchased from Cell Signaling. Antibodies can be seen in the table below.

| Primary Antibody | Company | Product No. |
|---|---|---|
| Cyclin A2 | Cell Signaling | 4656 |
| Cyclin B1 | Cell Signaling | 4135 |
| Cyclin D1 | Cell Signaling | 2978 |
| CDK4 | Cell Signaling | 12790 |
| CK19 | Proteintech | 10712-I-AP |
| EGRF | Cell Signaling | 4267 |
| p-EGFR | Cell Signaling | 2237 |
| Erk | Cell Signaling | 4695 |
| p-Erk | Cell Signaling | 4370 |
| GAPDH | Cell Signaling | 2118 |
| GSK3β | Cell Signaling | 5676 |
| p- GSK3β | Cell Signaling | 5558 |
| HNF4α | Perseus Proteomics | PP-H1415-00 |
| PNCA | Cell Signaling | 2586 |
| p-Rb | Cell Signaling | 8516 |
| Yap | Cell Signaling | 14074 |
| p-Yap | Cell Signaling | 4911 |

*Immunohistochemistry*

4 μm thick paraffin sections of liver were cut and PCNA, CK19 and H&E staining was performed as described previously (Borude, Edwards et al. 2012). H&E staining was done on a Leica Autostainer XL and CV5030 cover slipper. Briefly, slides stained for PCNA and CK19 were de-parafinized and boiled in citrate buffer for antigen retrieval. Antibodies were applied to the slides, and antibodies on slides were visualized using Vectastain ABC kit (Vector) and DAB peroxidase substrate kit (Vector). Slides were counter stained with hematoxylin and blued in Bluing Reagent (RICCA Chemical Company). All antibodies used for immunohistochemistry can be seen in the previous table.

*RNA-seq*

Total RNA was isolated from mouse liver tissue using TRIZOL. Mice from each group were pooled (n= 3-5) and sent to the KUMC genomics core facility for RNA-Seq analysis on an Illumina HiSeq 2000 (Illumina. San Diego, CA) and bioinformatics analysis was performed as previously described (Walesky, Edwards et al. 2013).

## 3.4 Results

*OGT KO Mice have an increased liver to body weight ratio after PHX*

Liver weight to body weight ratio of control mice plateaus at 7 days, while the OGT KO mice continues to increase to 14 days (Fig.3.4.1). This shows that OGT KO mice have an increase in liver regeneration when compared to control mice.

*OGT KO mice have a change in cell morphology*

H&E staining shows that OGT KO mice and control mice have similar liver morphology before PHX. However, at 14 days after PHX, the livers of OGT KO mice show an increase in vacuolization of cells as characterized by a loss of staining around the nuclei of cells (Fig.3.4.2).

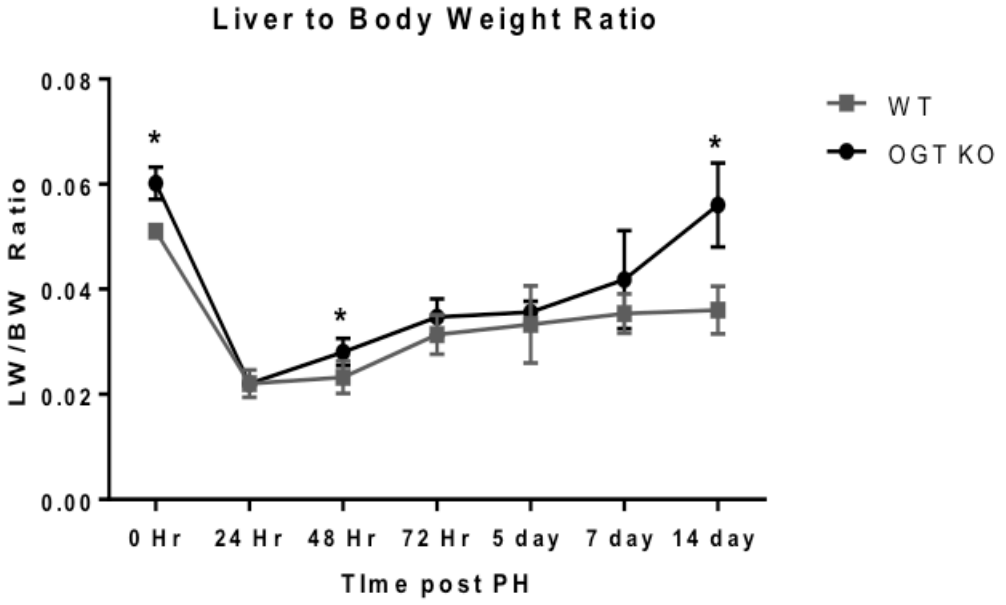



**Fig.3.4.1.** Liver to Body Weight Ratio. After PHX the liver regenerates to full mass on a time course of 0-14 days. OGT KO mice show increased liver regeneration compared to OGT WT mice. * Indicates statistical significant differences with a $p \leq 0.05$.

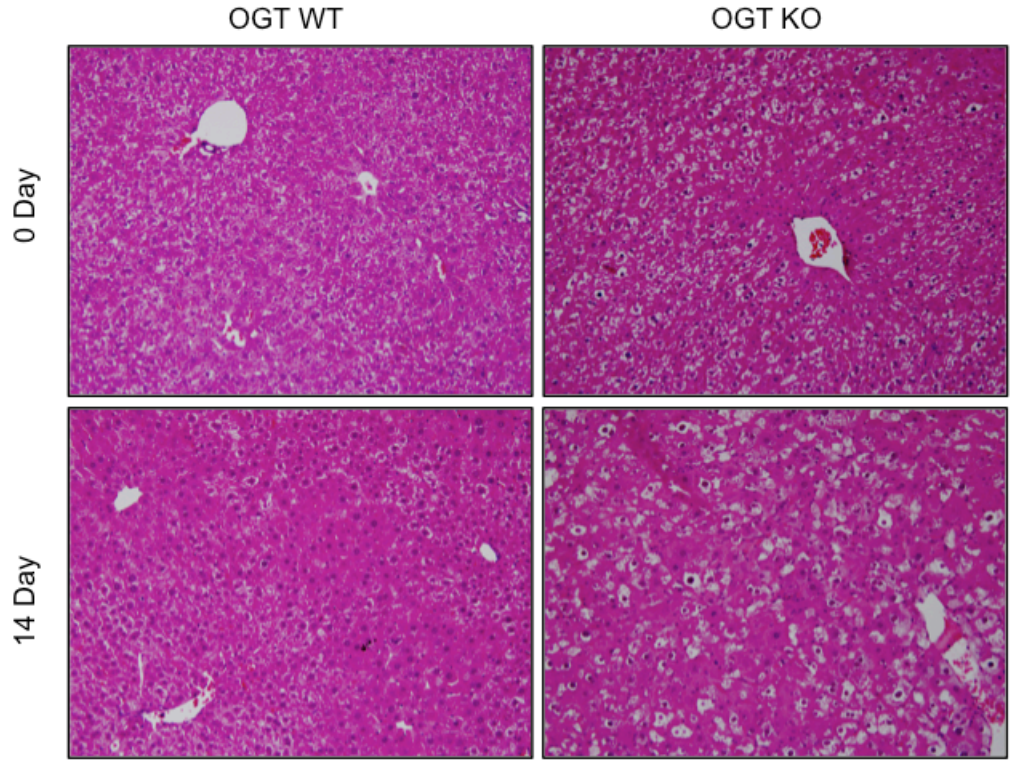



**Fig.3.4.2**. H&E immunohistochemistry. Time course of 0-14 days shows a change in cell morphology in the OGT KO mice at 14 days compared to OGT WT mice.

*Termination defect in OGT KO in liver regeneration after PHX*

In order to further examine the effects of OGT KO on liver regeneration after PHX, we examined several proteins critical in cell cycle progression and the regenerative response. Western blot analysis shows that Cyclin D1 expression was equal between WT and OGT KO mice up to 72 hr after PHX. However, OGT KO mice exhibited significantly higher Cyclin D1, A2 and B1 protein expression at 7 and 14 days when compared to WT mice (Fig.3.4.3). This was also observed with CDK4, pRb and PCNA expression. During the first 3 days (72 hr), these proteins were expressed at a comparable level in WT and OGT KO mice. However, the OGT KO mice had a significant increase in CDK4 at 14 days, in p-Rb at 5, 7 and 14 days and in PCNA protein at 5, 7 and 14 days after PHX (Fig.3.4.3). PCNA protein increase was also confirmed by immunohistochemistry showing an increase in PCNA positive cell at 7 and 14 days post PHX (Fig.3.4.4). Taken together, these data show that OGT KO mice have an extended cell proliferation after PHX, which is a hallmark of defective termination of liver regeneration.

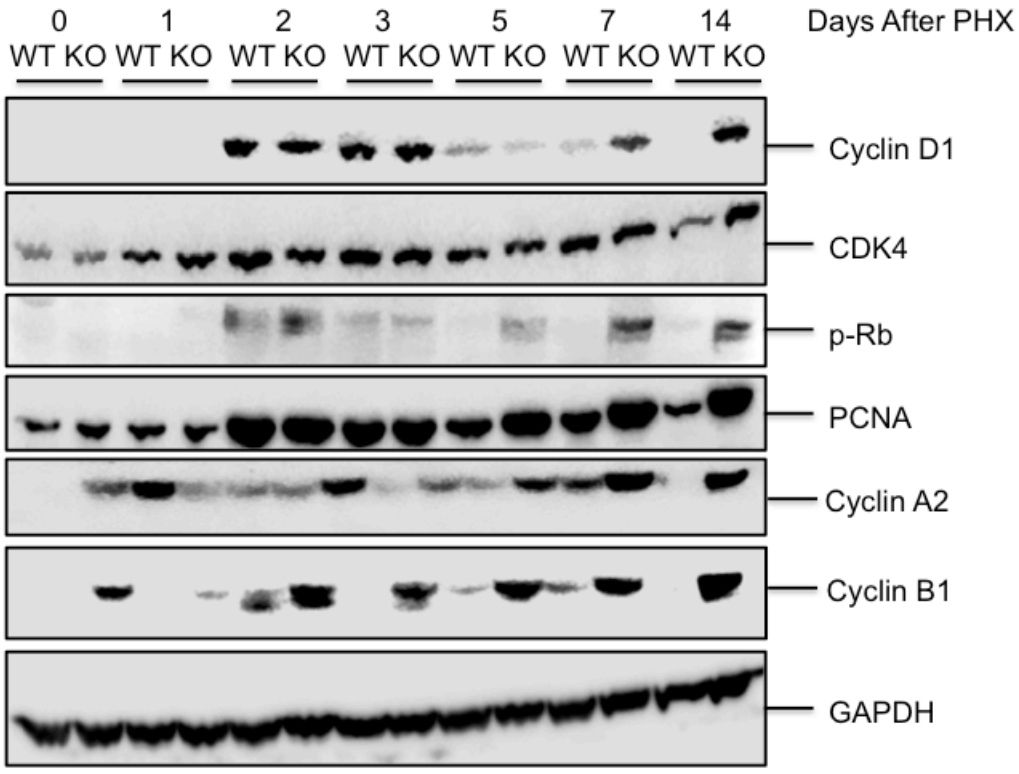



**Fig.3.4.3.** OGT KO leads to increased regeneration. Western blot analysis using total liver homogenate showing the increase in Cyclin D1, CDK4, p-Rb and PCNA after PHX in OGT KO mice.

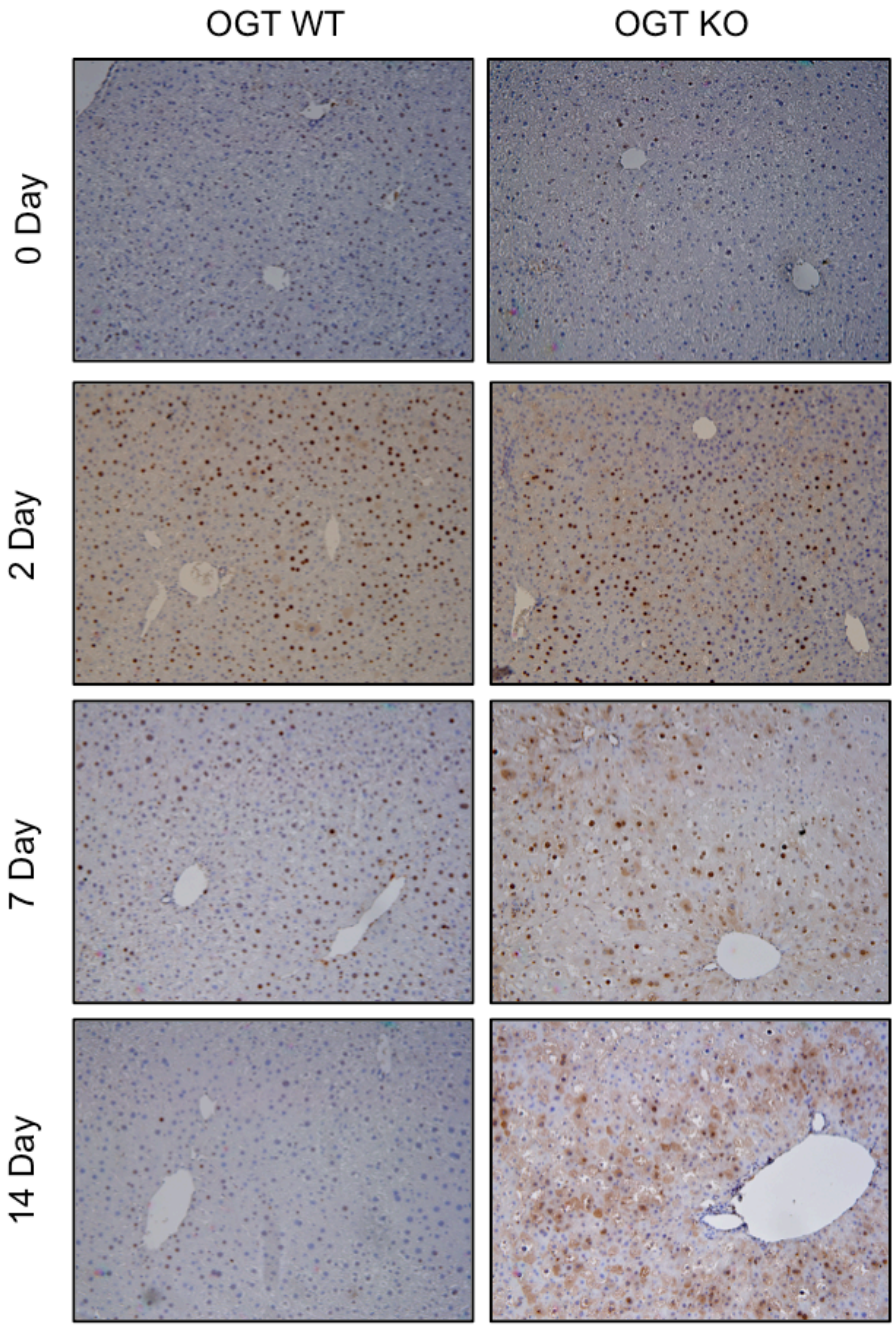



**Fig.3.4.4.** PCNA immunohistochemistry. Representative image of PCNA immunohistochemistry after PHX for OGT KO and OGT WT mice over a time course of 0-14 days.

*OGT KO mice have an increase in signaling associated sustained proliferation*

To determine the mechanisms of sustained proliferation in OGT KO mice after PHX, we determined changes in EGFR signaling, known to be a primary driver of hepatocyte proliferation (Michalopoulos 2007). Western blot analysis revealed that EGFR protein expression was elevated at 14 days in the OGT KO mice. Activation of EGFR as shown by p-EGFR was also increased at 14 days (Fig.3.4.5). Since we observed a marked defect in termination of regeneration, we investigated two other proteins involved in this process included Yes associated protein (YAP), the downstream regulator of Hippo Kinase pathway, and HNF4$\alpha$, the master regulator of liver differentiation. We observed that YAP activation as measured by ratio of total YAP to phosphorylated (inactive) YAP, is significantly higher in OGT KO mice at 14 days post PHX (Fig.3.4.5). Additionally, we observed a significant decrease in HNF4α protein expression in OGT KO mice at 14 days after PHX (Fig.3.4.5). All these changes in cell signaling are indicative of an increase in liver regeneration and a loss of termination signal for the liver in the OGT KO, leading to increased liver growth at 14 days. RNA-seq data shows an increase in fold change of most negative targets and a decrease in fold change of most positive targets of HNF4α at 14 days in OGT KO mice (Fig.3.4.6).

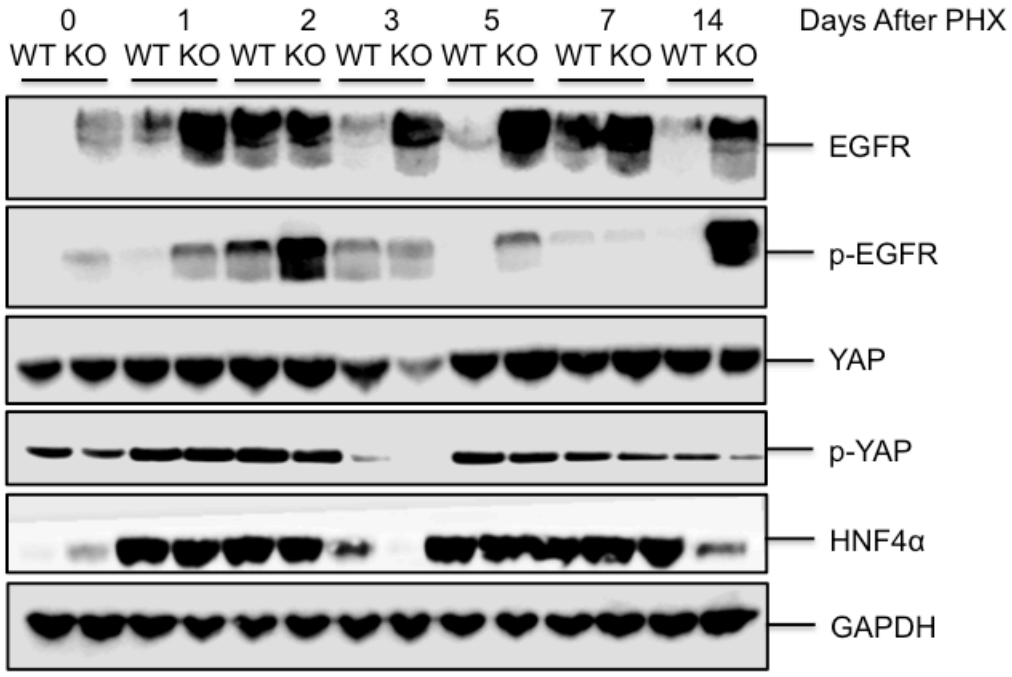



**Fig.3.4.5.** OGT KO leads to increased cell signaling. Western blot analysis of total liver protein lysates showing a variety of critical signaling proteins involved in liver regeneration in OGT KO and OGT WT mice over a time course of 0-14 days.

| Known Targets of HNFα | | |
|---|---|---|
| **Gene** | Positive/Negative Target | Fold Change |
| Slc34a2 | Negative | -2.0476 |
| Cdkn3 | Negative | Inf |
| Ect2 | Negative | 6.6782 |
| Egr1 | Negative | -37.4018 |
| Myc | Negative | -2.081 |
| Hjurp | Negative | 1.8411 |
| Akr1b7 | Negative | 13.4777 |
| Defb1 | Negative | 29.5417 |
| Acot3 | Negative | -10.4174 |
| Ccnd1 | Negative | 3.0961 |
| Ccna2 | Negative | 11.3706 |
| Ccnb1 | Negative | 33.6565 |
| F12 | Positive | -1.5701 |
| Cldn1 | Positive | 2.4317 |
| Apob | Positive | -1.4347 |
| Apoa2 | Positive | -3.7678 |
| Cyp2c37 | Positive | -4.7806 |
| Alas2 | Positive | -3.3307 |
| Ugt2b1 | Positive | -4.6461 |
| Dio1 | Positive | -19.3202 |
| Ces3a | Positive | -3.1024 |
| Ces3b | Positive | -13.1919 |
| F12 | Positive | -1.5701 |
| Ces3b | Positive | 2.4317 |

**Fig.3.4.6**. RNA-Seq fold change data of positive and negative targets of HNF4 $\alpha$ .

*OGT KO mice have an increase in CK19*

Histopathological analysis of livers from OGT KO mice at 7 and especially 14 days after PHX indicated presence of small cells with oval nuclei, which resembled oval cells. An oval cell is a term used to described hepatic progenitor cells, which normally do not contribute to liver regeneration. We further sought to determine activation of hepatic progenitors in OGT KO mice during PHX and stained paraffin sections for CK19, a marker used to identify both hepatic progenitors and cholangiocytes. OGT KO mice showed an increase in CK19+ cells at 14 days as shown by immunohistochemistry. OGT WT mice show CK19 only on the bile duct, While OGT KO mice have cellular staining of CK19 not associated with bile duct cells (Fig.3.4.7). The significance of these findings remains to be appreciated and significant additional studies are needed to delineate the role of hepatic progenitors in liver regeneration after PHX.

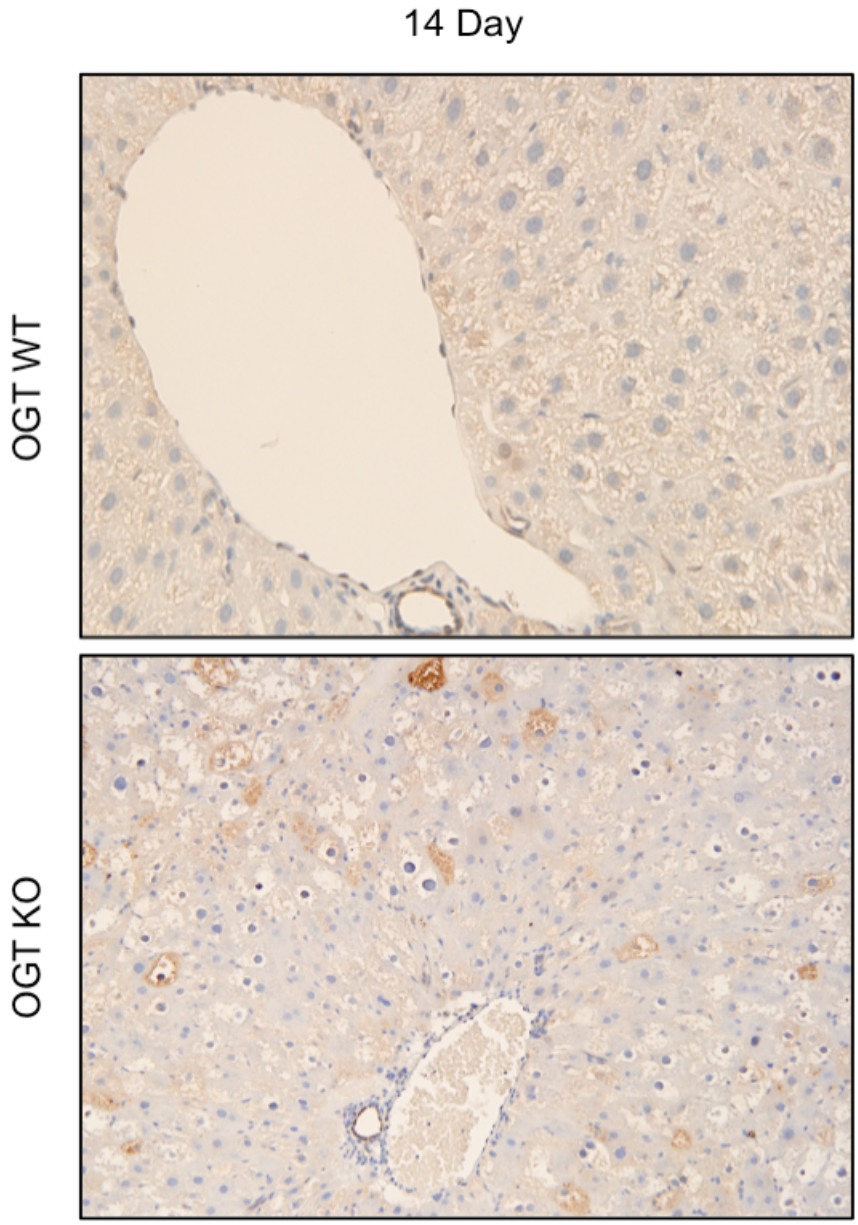



**Fig.3.4.7.** CK19 immunohistochemistry. A representative image of CK19 immunohistochemistry of OGT KO and OGT WT mice livers at 14 days.

## 3.5 Discussion

The liver has a remarkable ability to regenerate after partial resection and following drug induced liver failure (Michalopoulos and DeFrances 1997). In this study we utilize partial hepatectomy (PHX) model, where 60-70% of the liver is surgically resected to study effect of OGT deletion on liver regeneration. PHX allows the study of liver regeneration without necrosis or inflammation, making it an ideal tool to study regenerative organ growth (Michalopoulos 2010). The time course of signaling following PHX is precise and orderly, starting within the first few minutes post-PHX and continuing to 7-14 days after surgery (Michalopoulos 2007). The reproducibility and precise nature of the PHX model make it an ideal tool for studying liver regeneration. Interestingly, much is known about initiation of liver regeneration but the mechanisms of termination of liver regeneration are not well understood. Determining the factors involved in the termination of liver regeneration could hold the key to deciphering hepatocellular carcinoma (HCC) pathogenesis.

The deletion of OGT in mice, and the subsequent reduction of O-GlcNAcylation, results in increased hepatocyte proliferation and a loss in the termination signal of liver regeneration. Liver regeneration in OGT KO mice follows a similar pattern to OGT WT mice during the initial phase of liver regeneration up to 2 days post PHX. By 7 days there is change in cell morphology in OGT KO mice and cell proliferation continues until 14 days. These data demonstrate that decrease in O-GlcNAcylation by deletion of OGT results in

a termination defect following PHX and suggest a role for O-GlcNAcylation in inhibition of proliferation and re-differentiation of hepatocytes.

We also observed a decrease in HNF4α protein levels as shown by Western blot in OGT KO mice as compared to WT mice at 14 days following PHX (Fig.3.4.5). Previous studies have shown that HNF4α can suppress hepatocyte proliferation and a deletion of HNF4α can lead to increase proliferation and cancer (Bonzo, Ferry et al. 2012, Walesky, Gunewardena et al. 2012, Walesky, Edwards et al. 2013). Further, HNF4$\alpha$ is necessary not only to maintain hepatic differentiation but also to induce re-differentiation of hepatocytes following PHX (Huck, Manley Jr. et al. 2015, Huck, Manley Jr. et al. 2016). Decrease in HNF4$\alpha$ expression partially explains the termination defect in OGT KO mice.

We also observed a significant increase in YAP activity in OGT KO mice at 14 days after PHX. YAP is a known to help maintain organ size and is crucial for development (Zhao, Tumaneng et al. 2011). The phosphorylated form of YAP (p-YAP) is the inactive form of YAP and gets targeted for degradation. It has been shown previously that a loss of Mst1/2, the proteins that phosphorylate YAP, leads to liver overgrown and cancer (Zhou, Conrad et al. 2009). We observed a decrease in p-YAP in OGT KO mice indicating a suppression of Hippo/Yap signaling, which inhibits liver size expansion. Taken together these data point to OGT KO altering the dynamics of the termination phase of liver regeneration by promoting growth and suppressing the signal to terminate regeneration.

Recent studies have shown that EGFR signaling is essential for proper liver regeneration (Michalopoulos and DeFrances 1997, Paranjpe, Bowen et al. 2016). The ligands of EGFR, TGF$\alpha$ and EGF are primary mitogen of liver regeneration along with HGF (Block, Locker et al. 1996) and are critical in liver growth and regeneration (Mullhaupt, Feren et al. 1994). We observed that both EGFR and p-EGFR are elevated in OGT KO mice after PHX. This elevation of EGFR and p-EGFR is leading to an increase in liver regeneration after PHX. The fact that p-EGFR is elevated at 14 days post PHX in OGT KO mice also leads to the conclusion that the OGT KO mice liver is still growing. It is possible that several important proteins involved in signaling during liver regeneration and the termination of regeneration might be O-GlcNAcylated. In our model, the loss of O-GlcNAcylation due to OGT KO may be altering the activity and expression of these proteins leading to drastic changes in the signaling pathways important for liver regeneration.

In our study, we show that OGT KO, and thus a decrease in O-GlcNAcylation, leads to an increase in PCNA, Cyclins A2, B1 and D1 during termination phase of liver regeneration (7 and 14 days post PHX). RNA-seq data also showed an increase in fold change of the cyclins in OGT KO over WT. It is known that O-GlcNAcylation is an important regulator of the cell cycle and cell cycle progression. Elevation of O-GlcNAc was shown to reduce cell growth rates and lead to a delay in G2/M progression of the cell cycle in several cell lines, while decreased O-GlcNAc delays mitotic phosphorylation and alters cyclin expression (Slawson, Zachara et al. 2005). In another study, overexpression of

OGT was shown to decrease CDK1, the master regulator of M phase (Wang, Udeshi et al. 2010). These studies all show that the increase in O-GlcNAcylation leads to poor progression through the cell cycle and cell cycle delays. When coupled with our study showing a decrease in O-GlcNAcylation leads to an increase in cell cycle markers and cell proliferation, a clear role of O-GlcNAcylation and cell cycle progression is evident.

Taken together, the increase in pro-regenerative signaling and loss of termination signals result in sustained proliferation in OGT KO. These data show that OGT and O-GlcNAcylation are required for proper liver regeneration after PHX. This could prove to be of critical importance in HCC pathogenesis. It is known that signals that drive liver regeneration and those that drive proliferation in HCC are similar. However, pro-mitogenic signaling is precisely regulated and turned off at a specific time during liver regeneration thanks to effective termination and re-differentiation signals. In HCC, the pro-mitogenic signals remain active partly due to defect in termination signals. Our data indicate that loss of OGT function or decrease in O-GlcNAcylation could increase the likelihood on developing HCC. Whereas further research is necessary, this study shows that any alteration in the levels of O-GlcNAcylation can have profound effects on the health of the liver.

# Chapter 4- Discussion and Conclusions

O-GlcNAcylation is the covalent addition of a single N-acetyl glucosamine (O-GlcNAc) to a serine or threonine residue of a nuclear, cytoplasmic or mitochondrial proteins (Zachara, O'Donnell et al. 2004, Slawson, Copeland et al. 2010). O-GlcNAcylation is a dynamic intracellular process and, unlike other forms of glycosylation, there is no chain elongation (Slawson and Hart 2011). Despite the lack of a consensus sequence for binding, over 4000 protein are known to be O-GlcNAcylated (Ma and Hart 2014). O-GlcNAc has been implicated in many cellular processes and diseases including cell cycle regulation (Slawson, Zachara et al. 2005, Tan, Caro et al. 2013), cancer pathogenesis (Slawson and Hart 2011), and stress response (Zachara, O'Donnell et al. 2004). Despite the multitude of cellular processes that involve O-GlcNAc, very little work has been done on liver injury and regeneration. In this study, we examined the role of O-GlcNAc in acute liver injury after APAP and in liver regeneration after PHX.

It is known that approximately 50% of all the ALF cases in the developed countries are associated with overdose of APAP (Lee 1993, Lee 2003, Lee and Seremba 2008). Although APAP overdose is common, the mechanisms of APAP-induced ALF are not completely clear, because of which the treatment options for APAP-induced ALF remain extremely limited (Larson, Polson et al. 2005, Chung, Kim et al. 2006). Recent studies on the effects of post-translational modifications (PTMs) have demonstrated that PTMs of proteins such as sumoylation, lysine conjugation, methylation, ubiquitination, and O-GlcNAcylation play a central role in pathophysiology including stress response (Schrattenholz, Soskic et al. 2010). However, the role of many of these PTMs in APAP-induced

liver injury is currently not known. This lack of knowledge on the roles of PTMs represents a knowledge gap that needs to be bridged. In this study, we show that O-GlcNAcylation plays a central role in APAP induced liver injury and that modulation of O-GlcNAc levels not only has a profound effect on the safety of APAP but could also be used an intervention for APAP overdose.

In order to determine the role that O-GlcNAcylation plays in acute liver injury, we used the acetaminophen overdose model combined with both gain of function and loss of function approaches. The gain of function approach involved an increase in O-GlcNAcylation by pharmacological intervention using Thiamet-G (TMG), a potent inhibitor of O-GlcNAcase (OGA). The loss of function approach involved a decrease in O-GlcNAcylation using a hepatocyte specific deletion of O-GlcNAc transferase (OGT). In the TMG model (increased O-GlcNAcylation model) there was an increase in APAP-induced liver injury following both the acute and chronic treatment of TMG-mediated increased O-GlcNAcylation. Consistently, the OGT KO mice (the decreased O-GlcNAcylation model) showed a resistance to APAP induced liver injury. The decreased APAP-induced liver injury in OGT KO mice was accompanied by an increase in the ability of glutathione (GSH) recovery due to an up-regulation of antioxidant defense genes. Consistently, the expression of the GSH biosynthesis genes was decreased following TMG treatment. Further, our studies in collaboration with Dr. Slawson's group showed the modulation GSH biosynthesis genes by O-GlcNAcylation via changes in NRF2 expression. NRF2 is known to be the master

regulator of antioxidant response genes and the enzymes involved in GSH synthesis. (Wild and Mulcahy 2000, Enomoto, Itoh et al. 2001).

The results of our study show that there is a relationship with the level of O-GlcNAcylation and the susceptibility to APAP, and this relationship is mechanistically relevant to APAP toxicity. Our results show that OGT KO mice have an increased ability to recover glutathione (GSH) after APAP injury. GSH is an important molecule in response to oxidative stress. The importance of GSH to the recovery of APAP induced liver injury has been well established (Muldrew, James et al. 2002, Bajt, Knight et al. 2004). In the case of APAP, there is a massive increase in oxidative stress and mitochondrial damage that is observed in APAP injury (Jaeschke, McGill et al. 2012). When put together it is evident that O-GlcNAcylation is a crucial part of the response to APAP toxicity, and by controlling O-GlcNAcylation is may be possible to alter the profile of APAP toxicity. To date no work on APAP toxicity has been performed using OGA KO mice (Keembiyehetty, Love et al. 2015), but this could provide even more evidence that O-GlcNAcylation is critically important for the safety of APAP.

O-GlcNAcylation is an essential cellular process. The deletion of either OGT or OGA, the enzymes that regulate O-GlcNAcylation is embryonic lethal (Shafi, Iyer et al. 2000, Keembiyehetty, Love et al. 2015). Previous studies have shown that O-GlcNAc is increased in response to heat stress (Sohn, Lee et al. 2004, Zachara, O'Donnell et al. 2004), ischemia reperfusion injury in the heart (Champattanachai, Marchase et al. 2008, Jones, Zachara et al. 2008) and inflammation in the heart (Xing, Feng et al. 2008). These studies all point to a

protective pro-survival role of increased O-GlcNAcylation in response to stress, but our results show that an increase in O-GlcNAcylation exacerbates APAP induced liver injury. A reason for the apparent contradiction could be that the effects of O-GlcNAcylation are context specific; meaning alterations in O-GlcNAcylation profile might be dependent on not only the type of stress but also the stressor. The fact that our results are paradoxical to what is known about O-GlcNAcylation and other stress responses makes it clear that this more work needs to be done to understand the role of O-GlcNAcylation in APAP overdose.

The other physiological process significantly affected by O-GlcNAcylation is cell proliferation. However, the role of O-GlcNAcylation in regulating cell proliferation in the liver is not known. We investigated the effects of decreased O-GlcNAcylation on liver cell proliferation using the partial hepatectomy (PHX) model in the OGT KO mice. Liver regeneration after PHX is a well-studied model and it involves matrix remodeling, growth factor signaling and hepatocyte proliferation (Michalopoulos and DeFrances 1997). Since the events occur in an orderly and predictable fashion, it is easy to examine the effects of different factors on the overall ability of the liver to regenerate after PHX. Our studies showed that decrease in O-GlcNAcylation results in continued cell proliferation in the liver well beyond the 3-5 day post-PHX window observed in WT mice. The increase in liver size and the preponderance of signaling molecules important for liver regeneration that are unregulated after PHX in OGT KO mice stress that O-GlcNAcylation is essential for effective termination of liver regeneration. Due to the large increase in regeneration after OGT KO this study also provides

evidence that modulation of O-GlcNAc could be a potential therapeutic intervention for cancer, possibly by treating with the potent OGA inhibitor TMG. A potential future direction would be to perform OGT KO and OGA KO and induce hepatocellular carcinoma (HCC) with the treatment of diethyl nitrosamine.

There is a potential that key signaling molecules important for proper liver regeneration are in fact O-GlcNAcylated, which in turn would regulate their activity. Previous studies have shown that O-GlcNAcylation of proteins is critical to their function, by either promoting activation or ensuring deactivation. In pancreatic cancer cells, hyper-O-GlcNAcylation was shown to increase the activity of NFκB p65 subunit and the upstream kinases IKKα and IKKβ, while reducing hyper-O-GlcNAcylation lead to loss of p65 nuclear translocation and subsequent target gene expression (Ma, Vocadlo et al. 2013). NFκB was shown to be required for hepatocyte proliferation after PHX in mice after a deletion of NEMO, a modifier of NFκB, and this also lead to an increase in oxidative stress; but there was compensatory regeneration from hepatic oval cells (Malato, Ehedego et al. 2012). The loss of NFκB through a targeted deletion of its upstream kinase IKKβ triggers the immune response and leads to earlier cell proliferation during liver regeneration (Malato, Sander et al. 2008). NFκB is just one of many proteins important for liver regeneration whose function is controlled by O-GlcNAcylation. Determining the role O-GlcNAc plays in the signaling during liver regeneration represents an untapped frontier in liver regeneration, and O-GlcNAcylation of NFκB is just one signaling protein that can be examined in the

context of liver regeneration after either an increase or decrease in protein O-GlcNAcylation in the future.

We observed a significantly sustained and up regulated proliferative response following PHX in OGT KO mice. In the cell cycle, E2F family of transcription factors is essential to transition the cell from G1 to S phase of the cell cycle. In order for E2F to be activated, Cyclin D1 must bind CDK4 which in turn phosphorylated Rb protein leading to the inactivation of Rb (Dimova and Dyson 2005). Previous studies have shown that Rb function is controlled by O-GlcNAc (Wells, Slawson et al. 2011) and that and the overexpression of E2F-1 negatively regulates OGT (Muthusamy, Hong et al. 2015). Overexpression of OGT was shown to decrease CDK1 activity, the master regulator of M-phase progression (Wang, Udeshi et al. 2010). O-GlcNAcylation, or more specifically the proper balance of O-GlcNAcylation, is immensely important for cell cycle progression. It was shown previously that overexpression of either OGT or OGA causes disruption in cyclin periodicity and causes improper cytokinesis (Slawson, Zachara et al. 2005). These studies point to an integral role of O-GlcNAc in maintaining the cell cycle. In the context of OGT KO, it should not be surprising that loss of OGT and subsequent ablation of O-GlcNAcylation leads to aberrant liver regeneration after PHX. In our study, there was a significant and sustained increase in Cyclin D1, A2, B1 and p-Rb, as well as a large increase in PCNA positive cells in OGT KO mice, which is evidence of an increase flux through the cell cycle.

Our study also shows there is an increase in CK19 at 14 days post PHX. In the normal human liver CK19 is expression in the bile duct epithelium (Lemaigre 2015). Expression of CK19 is also seen in hepatic progenitor cells or oval cells (Wang, Foster et al. 2003). The existence of CK19 staining in OGT KO livers outside of the biliary epithelium suggests that there might be oval cell or biliary cell expansion and proliferation in the livers of OGT KO mice after PHX, with a potential outcome being cancer. Future studies are needed to examine the role of the presence of CK19 outside the biliary epithelium in OGT KO livers and determine if OGT KO alters the bile duct and bile flow, or if prolonged regeneration leads to liver cancer due to OGT KO.

O-GlcNAcylation of transcription factors has been shown to influence their activity, DNA binding, stability and interaction with co-factors (Ozcan, Andrali et al. 2010). The O-GlcNAcylation of p53 has been shown to decrease phosphorylation and subsequent ubiquitination, leading to a more stable p53 by blocking proteolysis (Yang, Kim et al. 2006). As a known tumor suppressor, p53 plays a critical role in stabilizing cells under stress due to its anti-proliferation capacity. In our OGT KO model of liver regeneration there is prolonged hepatocyte proliferation, with the number of PCNA positive cells remaining increased at 14 days post PHX. This suggests that there might be a role for p53 in the OGT KO model of liver regeneration where p53 is suppressed in OGT KO mice during liver regeneration; but future studies would need to be conducted to confirm a role for p53.

Overall, our studies show that alterations in O-GlcNAcylation play an important role in both drug-induced liver injury after APAP overdose and liver regeneration after PHX. The work done provides insight into an area of study that has not been studies previously, and shown a new role for O-GlcNAc. It also points to the importance of maintaining appropriate levels of protein O-GlcNAcylation for proper regulation of protein function. The work done here has provided an insight into the role of O-GlcNAc in APAP induced liver failure that could potentially lead to a therapeutic intervention. A potent inhibitor of OGT could potentially be used as a treatment for APAP in conjugate with N-acetylcysteine. We have also highlighted the role of O-GlcNAc in liver regeneration and shown that a decrease in O-GlcNAcylation leads to a loss of liver regeneration termination signal and an increase in hepatocyte proliferation. These data could have a profound effect on how we view cancer pathogenesis. It might be possible to treat HCC patients with TMG, a potent OGA inhibitor, and slow down the progression of cancer. Although this study points to a role in O-GlcNAcylation in many signaling cascades after PHX, the exact mechanisms are not known. An important future direction for this project would be to determine which pathways are being up regulated or down regulated in the OGT KO mice during regeneration. Another important future direction would be to determine the role of OGT KO on the metabolism of glucose and glycogen storage in the liver following both APAP induced liver injury and liver regeneration after PHX. This would lead to a more complete understanding of the role of O-GlcNAcylation in

the liver. In conclusion, this study has shown a novel role for O-GlcNAc in the liver, and stressed the importance of maintaining a balance in O-GlcNAcylation.

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